DEVELOPMENT AND EVALUATION OF DOUBLE GENES TARGETED MULTIPLEX PCR ASSAYS FOR THE DETERMINATION OF BOVINE, BUFFALO AND PORCINE MATERIALS IN FOOD PRODUCTS

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ABSTRACT

Bovine, buffalo and porcine materials in food products are sensitive to religions and a big threat to health and fair economic practices. Current methods to authenticate these animal materials in food chain are based on mainly single gene target which are generally longer in length and thus breakdown during food processing treatments. For the first time, here I targeted double gene sites in short-amplicon length multiplex polymerase chain reaction (mPCR) assays for the detection and differentiation of bovine, buffalo and porcine materials in food chain. Multiple targets detection in single assay saves analytical cost and time. Both the conventional and real-time PCR platforms were developed and authentic target detection was confirmed through sequencing and Restriction Fragment Length Polymorphism analysis. Mitochondrial cytochrome b (cytb) and NADH dehydrogenase subunit 5 (ND5) genes were targeted and six different targets (length: 73-146 bp), two for each of cow (121 and 106 bp), buffalo (90 and 138 bp) and pig (73 and 146 bp), were amplified from raw, boiled, autoclaved and microwaved cooked meat under pure and mixed matrices. The specificity of the PCR assays were checked against three targets and 25 non-target species. Specific PCR products were found only from beef, buffalo, and pork that were targeted in this assay. To eliminate the possibility of any false-negative detection, eukaryotic endogenous control was used for specificity testing. The detection limit was 0.01 ng DNA for tetraplex and 0.02 ng DNA for hexaplex under pure states and 0.1% target meat in mixed and commercial matrices. Complete sequence matching was found for five the PCR products but 98.5% for buffalo ND5 gene. The PCR products were digested by four restriction enzymes, namely *Alu*I, *Eci*I, *Fat*I and *CviKI*-1 and clear restriction fingerprints were obtained. The developed methods were used for the screening of bovine, buffalo and porcine materials in various commercial meat curries and processed foods, namely, meatballs and frankfurters. Survey results revealed about 80% of beef meatballs were adulterated with buffalo and about 20% of beef products were

totally replaced with buffalo. Moreover, the analysis of 20 beef frankfurters revealed the presence of both beef and buffalo in all specimens. This demonstrated that all beef frankfurter products are adulterated with buffalo in Malaysia. However, the analysis of 7 beef curries reflected only 2 them were beef and 5 were buffalo. In contrast, porcine meatball and frankfurter were found 100% authentic and also no pork was detected in halal branded beef curries, meatballs and frankfurters and chicken frankfurters. Finally, the developed TaqMan probe multiplex real-time PCR (mqPCR) assay successfully detected 0.003 ng DNA in a pure state and 0.1% target meat in mixed and commercial matrices. Analysis of commercial products under mqPCR assay revealed 71% and 100% of beef frankfurters, meatballs and 85% burgers contained buffalo adulteration but no pork in Malaysian markets. The advantage of the method was evidenced in terms of fidelity, cost and time since all the three species were detected and the option of alternative targets could complement missing targets even in decomposed specimens.

ABSTRAK

Daging lembu, kerbau dan babi di dalam produk makanan menyentuh sensitiviti agama dan merupakan ancaman yang besar terhadap kesihatan dan ekonomi yang saksama. Kaedah semasa untuk mengesahkan bahan-bahan haiwan dalam rantaian makanan adalah berfokus kepada sasaran gen tunggal yang lazimnya lebih panjang, maka kerosakan pada gen kerap berlaku semasa pemprosesan makanan. Buat julung kalinya, saya menyasarkan dua lokasi gen dengan amplikon berjarak pendek dalam penilaian rantaian tindak balas polimeras (PCR) multipleks untuk mengesahkan pengesanan dan pembezaan daging lembu, kerbau dan babi dalam rantai makanan. Pengesanan pelbagai sasaran dalam penilaian tunggal dapat menjimatkan kos analisis dan masa. Kedua-dua platform PCR konvensional dan masa-nyata telah digunakan dan pengesanan sasaran dipastikan melalui penjujukan dan analisis Polimorfisma Panjang Fragmen Terbatas. Gen mitokondria Cytochrome b (cytb) dan NADH dehydrogenase sub unit 5 (ND5) telah disasarkan, dan enam sasaran yang berbeza (panjang: 73-146 bp), dua untuk lembu (121 dan 106 bp), kerbau (90 dan 138 bp) dan babi (73 dan 146 bp), telah diamplifikasikan daripada daging mentah, direbus, diautoklaf dan dimasak melalui gelombang mikro, sebagai daging semata ataupun campuran pelbagai matriks. Spesifikasi asai PCR ditentukan ke atas tiga sasaran dan 25 spesis bukan sasaran.Ujian spesifik produk PCR hanya dijumpai pada daging lembu, daging kerbau dan daging babi yang menjadi sasaran di dalam asai ini. Untuk menyingkirkan pengesanan salah-negatif, kawalan endogenous eukaryotik telah digunakan dalam ujian spesifik. Had pengesanan adalah 0.01 ng DNA untuk tetrapleks dan 0.02 ng DNA ng untuk heksapleks dalam keadaan tulen dan 0.1% daging sasaran dalam matriks bercampur dan matriks komersil. Penjujukan sempurna dikesan untuk lima produk PCR namun hanya 98.5% untuk gen ND5 bagi daging kerbau. Produk PCR telah dicerna oleh empat enzim terbatas, iaitu *Alu*I, *Eci*I, *Fat*I dan *CviKI*-1 dan cap jari terbatas jelas diperolehi. Pembangunan kaedah ini telah digunakan untuk

menganalisis lembu, kerbau dan babi dalam pelbagai kari daging komersial dan makanan yang diproses, termasuk bebola daging dan frankfurter. Keputusan kaji selidik mendedahkan kira-kira 80% daripada bebola daging lembu telah dicemari dengan kerbau dan kira-kira 20% produk daging lembu telah sama sekali digantikan dengan kerbau. Selain itu, analisis 20 frankfurter daging lembu mendedahkan kewujudan kedua-dua daging lembu dan kerbau dalam semua spesimen. Ini menunjukkan bahawa semua produk frankfurter daging lembu dicemari kerbau di Malaysia. Walau bagaimanapun, analisis 7 kari daging lembu menunjukkan hanya 2 daripada sampel adalah daging lembu manakala 5 adalah kerbau. Sebaliknya, bebola dan frankfurter babi didapati 100% asli dan tiada daging babi dikesan dalam kari daging lembu berlabel halal, bebola daging dan frankfurter serta frankfurter ayam. Akhirnya, prob Taqman multipleks asai PCR masa-nyata yang telah dibangunkan berjaya mengesan 0.003 ng DNA dalam keadaan tulen dan 0.1% daging sasaran di dalam matriks campuran dan komersial. Analisis produk komersial menggunakan asai mqPCR mendedahkan 71% dan 100% frankfurter daging lembu, bebola daging dan 85% burger mengandungi penambahan daging kerbau tetapi tiada pengubahsuaian menggunakan daging babi di dalam pasaran Malaysia. Kelebihan kaedah ini dibuktikan melalui ketepatan, kos dan masa kerana ketiga-tiga spesis berjaya dikesan dan sasaran alternatif dapat melengkapkan sasaran yang hilang walaupun di dalam spesimen terurai.

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$	:	dollar
%	:	percent
^{0}C	:	degree celsius
µg	:	microgram
µL	:	microliter
µM	:	micromole
pg	:	Picograme
≥	:	greater than or equal to
3D	:	three dimensional
A260/A280	:	ratio of absorbance at 260 and 280 nm
AIDS	:	acquired immune deficiency syndrome
BLAST	:	Basic local alignment search tool
bp	:	base pair
BSP	:	bovine spongiform encephalopathy
Bucytb	:	buffalo cytb
BuND5	:	buffalo ND5
COI	:	cytochrome c oxidase subunit I
Cocytb	:	cow cytb
CoND5	:	Cow ND5
Ct	:	threshold cycle
cytb	:	cytochrome b
dH2O	:	distilled water
D-loop	:	displacement loop
DNA	:	deoxyribonucleic acid

dsDNA	:	double stranded- deoxyribonucleic acid
EC	:	European Commission
ELISA	:	enzyme-linked immunosorbant assay
FAO	:	Food and Agriculture Organization
FDA	:	Food and Drug Administration
FTIR	:	fourier transformed infrared
g	:	gram
GC-MS	:	gas chromatography-mass spectrometry
GMP	:	good manufacturing practice
h	:	hour
HACCP	:	hazard analysis and critical control points
HEV	:	hepatitis E virus
HIV	:	human immunodeficiency virus
HPLC	:	high performance liquid chromatography
IDT	:	integrated DNA technology
IFE	:	isoelectric focusing
LOD	:	limit of detection
MEGA5	:	molecular evolutionary genetics analysis version 5
mg	:	milligram
$MgCl_2$	:	magnesium chloride
min	:	minute
ml	:	milliliter
mM	:	milimolar
mPCR	:	multiplex polymerase chain reaction
mqPCR	:	Multiplex real-time PCR
mt	:	mitochondrial

NCBI	:	national center of biological information
ND5	:	NADH dehydrogenase subunit 5
ng	:	nanogram
nt	:	nucleotide
nDNA	:	Nuclear DNA
OECD	:	Organization for Economic Cooperation and Development
PCB	:	polychlorinated biphenyl
PCR	:	polymerase chain reaction
Pocytb	:	pig cytb
PoND5	:	pig ND5
RFLP	:	restriction fragment length polymorphism
psi	:	pounds per square inch
RAPD	:	randomly amplified polymorphic DNA
rRNA	:	ribosomal ribonucleic acid :
s	:	second
ssPCR	:	species specific PCR
Ta	:	annealing temperature
Tm	:	melting temperature
UN	:	united nation
USDA	:	US department of Agriculture
UV	:	ultraviolet
w/w	:	weight/weight
IAC	:	internal amplification control

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CHAPTER 1: INTRODUCTION

1.1 Background of the Study

Authentication of the species origins of animal-originated food products is a rapidly growing field because of its direct relevance to public health, biodiversity perspectives as well as people's religious and cultural traditions. Increasing public awareness about the roles of foodstuffs in maintaining community health and limit the spread of both communicable and non-communicable diseases along with their roles in balanced economy and religious understanding has further contributed to the identification of animal sources in foods and feeds (Bottero & Dalmasso, 2011; Haider, Nabulsi, & Al-Safadi, 2012). In fact, it is an integral requirement to safeguard community health, religious faith, fair trade and consumers' lifestyles. Nowadays, the consumption of ready-made foods from the nearby food court, restaurant and groceries has dramatically increased due to the increasing volumes of works and extension of business hours. These are making people dependent on ready-made foods, such as burgers, frankfurters, meatballs, pizzas, cookies, soups, creams, candies and restaurant foods (Ali, Razzak, & Hamid, 2014c). Therefore, the consumers' are paying great attention to the quality and ingredients of ready-made and packaged foods due to the health concern (e.g. Zoonotic threats and allergens), lifestyles (vegetarianism and organic food), religious faith (e.g. Ritually prohibited ingredients) and fair spending of their hard earned fortunes (Ali et al., 2015d).

However, the unexpected occurrence of certain adulterants in food such as horse meat in school meals (Richardson, 2013), burger products in Europe (Walker, Burns, & Burns, 2013), rat meat sold as lamb in China (Ali et al., 2014c), monkey meat in soup in Indonesia (Rashid et al., 2015b) and cat meat in Indian curries in Britain (Ali, Amin, Hamid, Hossain, & Mustafa, 2015a) has made customers are increasingly worried about

the religion compliant foods. The recent inclusion of some alien species pork and rat meat in lamb products (Ali et al., 2014c), monkey and dog meat in soup products (Rahman et al., 2014; Rashid et al., 2015b), dog and cat meat for chevon (Singh, Pathak, Nayak, Verma, & Umaraw, 2014) is of grave concern and highly alarming since most of these species are not only the potential carrier of infectious zoonoses but also they are prohibited in several religions such as Islam and Judaism. In Europe, the consumption of beef has fallen drastically due to the bovine spongiform encephalopathy (BSE), polychlorinated biphenyl (PCB) or toxic dioxin contamination, swine influenza and avian influenza in 2010 (Goffaux, China, Dams, Clinquart, & Daube, 2005; Bottero & Dalmasso, 2011). Researchers believe that Simian Immunodeficiency Virus affected African chimpanzee meat is responsible for the most fatal and infectious human disease, HIV/AIDS (Fajardo, Gonza´lez, Rojas, Garcıa, & Martı´n, 2010). Furthermore, religious taboos are also dominant issues to choose and avoidance of food products, particularly meat products (Ali et al., 2014c).Thus, the proper labeling of constituents in food products and their subsequent field monitoring has become a need of the time to prevent food forgery, safeguard consumers trust and sustainable food businesses. Considering the need, most of the countries have regulatory bodies for the tracing and tracking of adulterants such as lower grade or lower priced meats in the higher priced meat products (Al Amin, 2015).

Moreover, protecting the sanctity of halal branded food products has become a global issue because of the rapid expansion of halal food markets in all corners of the world (Ali et al., 2015d; Rahman et al., 2014). Currently, the Muslim population has reached to 1.8 billion (HKTDC, 2014) and turnover of the global halal business has crossed to US$ 2.3 trillion in 2012 and it is going to be US$ 2.47 trillion by 2018 (HKTDC, 2014). Customers pay more value for halal foods because of its special requirements of manufacturing and supply chain (Ali et al., 2015d) which have made them susceptible to adulteration. Thus

in order to adapt and grab the huge opportunities of global halal food markets, many countries such as Malaysia, Singapore, Thailand, Indonesia, India, Turkey, Australia, China, New Zealand, Brunei and Brazil have established halal certification and regulatory bodies (Ali et al., 2012d, Salama, 2011). The Malaysian government has developed more than 10 integrated Halal hubs to monitor and export halal goods to other countries. In 2013, Malaysia was the global largest exporter of halal products and the total turnover was US$ 10 billion (HKTDC, 2014). Thus halal food industry has been evolved as an important contributor to Malaysian economy and Malaysia must protect the sanctity of this industry. Modern food products such as meatballs, frankfurters and burgers are made from minced meats and detection of the animal origin of those samples are extremely difficult due to the complexity of the matrices. Therefore, the development and validation of the developed methods need the screening of real-world samples to be sure that they are working in the field.

Up-to-this-date, various analytical approaches have been documented to detect fraudulent mixing of food products. Numerous lipids (Rohman, Erwanto, & Man, 2011), proteins (Ayaz, Ayaz, & Erol, 2006) and DNA-based assays have been proposed for meat speciation (Matsunaga et al., 1999, Ali et al., 2015b). However, the lipid and protein based methods are often unsuitable because they are laborious, target-biomarkers are often modified and thus cannot distinguish closely related species in highly processed food such as heated or chemically treated products, and are less sensitive than DNA-based approaches (Ali et al., 2012b; Lago, Herrero, Madriñán, Vieites, & Espiñeira, 2011). Moreover, these methods are unable to differentiate closely related species, such as cow and buffalo. In contrast, the DNA-based techniques, especially the short-length DNA biomarkers are thermodynamically more stable, more sensitive and more reliable over the longer ones even under extreme states such as degraded or naturally decomposed samples (Ali et al., 2015b; Rashid et al., 2015b). Among the DNA-based methods, PCR

approaches are highly appreciated since they can amplify target biomarkers from single copy to easily detectable quantities, offering a highly sensitive, robust and low-cost platform for the identification of biological ingredients (Rashid et al., 2015b). Several PCR-platforms such as conventional includes species-specific singleplex PCR (Karabasanavar et al., 2011; Rashid et al., 2015b), multiplex PCR (Ali et al., 2015d; Bottero et al., 2003), PCR-restriction fragment length polymorphism (PCR-RFLP) (Ali, Hashim, Mustafa, & Man, 2011b; Dooley et al., 2005), randomly amplified polymorphic DNA (RAPD) (Arslan, İlhak, Calicioglu, & Karahan, 2005), PCR product sequencing (La Neve, Civera, Mucci, & Bottero, 2008), and real-time PCR includes TaqMan probe (Ali et al., 2012a; Drummond et al., 2013), SYBER green (Asing et al., 2016a), Eva green and molecular beacon, sensor based includes nucleic acid based biosensor and nanoparticle based biosensor have already been documented for the authentication of meat species.

However, these methods are mostly based on a single and a long DNA marker which often breaks down under food processing treatments as well as natural and environmental decomposition, making them less trustworthy but more expensive (Ali et al., 2015c). The evolution of multiplex polymerase chain reaction (mPCR) assays are especially promising since they offer the opportunity of identifying multiple target oligos in a single assay platform, saving both analytical cost and time (Ali et al., 2014c). Instead of targeting a single gene, double gene targeting short-amplicon length mPCR assays would be more reliable and trustworthy because of the complementation opportunities in which the detection of an alternative target can compensate the missing target, in case it is lost under the states of decomposition. Moreover, the species-specific PCR restriction fragment length polymorphism (PCR-RFLP) assays are especially interesting because they offer the opportunity to authenticate a product by restrictive digestion of the amplified PCR products using one or more restriction enzymes (REs) (Rashid et al.,

2015b). Using the sequence variation that exists within a defined region of DNA, the differentiation of even closely related species is possible using a PCR-RFLP assay. In contrast to conventional PCR assays, real-time PCR techniques are especially promising since they offer the opportunity of fast, greater resolution, target quantification, automation, reproducibility, high sensitivity and real-time monitoring (Asing et al., 2016a; Cheng, He, Huang, Huang, & Zhou, 2014). In addition, TaqMan probe based real-time PCR method is particularly beneficial since specifically-designed probe and primer sets significantly enhance the specificity and reliability of the assay (Ali et al., 2012a). Because fluorescent signal is generated only when hybridize the specific probe due to the DNA polymerase moves by and cleaves off the probe's quencher molecule (Arya et al., 2014). Furthermore, TaqMan probe based techniques significantly facilitate to develop the multiplex real-time PCR assays because specific probes can be labeled with distinguishable and different reporter dyes which allows the identification of amplifications formed by one or multiple primer sets in a single PCR assay tube. The main drawback of the DNA-intercalating dye systems (SYBER green, Eva green) are that bind non-specifically to all double-stranded DNAs produced during the PCR reaction such as primer-dimers or any non-specific products, resulting in increased fluorescent background or false positive (Arya et al., 2014). Moreover, some dyes are known to inhibit the PCR reaction (Gudnason, Dufva, Bang, & Wolff, 2007).

Mitochondrial genes were targeted for the design of species-specific primers since they are maternally inherited, single allele by nature and there is no possibility in sequence ambiguities and present in multiple copies per cell. These ensure a higher probability of getting the desired PCR results even in case of serious DNA breakdown under harsh processing conditions (Mane et al., 2012b). Ad-mixture of closely related animal species can easily be discriminated by virtue of the larger variety of mitochondrial DNA (mtDNA) over the nuclear sequence (Gupta, Rank, & Joshi, 2011). Due to the presence

of variable and conserved regions, cytb gene is also suitable for phylogenetic studies because of the divergence at the population level, explaining the evolutionary relationship (Mohamad, El Sheikha, Mustafa, & Mokhtar, 2013). On the other hand, ND5 gene contains sufficient conserved regions within the same species, but adequate polymorphism among the closely related species (Ali et al., 2015d).

In this regard, mPCR assay, especially the double gene targeting one with short amplicon targets, would be especially useful and trustworthy for the simultaneous detection of beef, buffalo, and pork products in various food products. Hence, all of the documented PCR assays involving single and longer gene target, are definitely less reliable than a short-length double gene-target PCR assay where dual targets complements each other, offering a confirmed and unambiguous detection.

1.2 Project Rationale

Beef, buffalo and pork are economically and culturally important meat having the top rate of consumption in most parts of the world. Religious, cultural, and geographical restrictions and preferences over the consumption of beef, buffalo, and pork are huge, and social outcry over their adulteration and consumption have taken place from time to time (Girish, Haunshi, Vaithiyanathan, Rajitha, & Ramakrishna, 2013; Karabasanavar et al., 2011). While Egyptians prefer buffalo because of their cultural preferences, some Europeans and Indians avoid beef because of the fear of bovine spongiform encephalopathy (BSE) and religious requirements (Sakaridis, Ganopoulos, Argiriou, & Tsaftaris, 2013), beef has evolved as the meat of choice and consumed heavily in most parts of the world (Karabasanavar et al., 2011a; Karabasanavar et al., 2011b). For instance, while there is a huge turnover of buffalo in India both for domestic consumption and exports, the slaughtering, consumption and export of beef is totally prohibited there because of the restriction of Hinduism (Girish et al., 2013). The recent killing of a man

and beating of his family members in the city of Dadri in India by a group of radical Hindus based on a false rumor that beef was kept in his refrigerator is a strong piece of evidence that meat scandal can provoke social unrest and take innocent lives (Matthew, 2015). On the other hand, pork is totally unacceptable to the Muslim, Jewish and select Christian dominations despite its popularity in Western countries (Ali et al., 2012a; von Bargen, Dojahn, Waidelich, Humpf, & Brockmeyer, 2013). Therefore, beef, buffalo and pork adulterated meat products have direct implications to public health, religions, cultures and economy. Major risks include zoonotic infection, financial loss and social outburst. Bovine and porcine products are directly linked to the fatal neurodegenerative disease bovine spongiform encephalopathy (BSE) and dioxin induced developmental, reproductive and carcinogenic disorders (Bottero & Dalmasso, 2011). Porcine contamination can further lead to infections caused by swine influenza (Bottero & Dalmasso, 2011) and *Toxoplasma gondii* (Robert-Gangneux & Dardé, 2012). Buffalopox is an important zoonotic infection speed by the *Orthopoxvirus* infected buffalo and cow (Gurav et al., 2011). Thus, the social, religious, health, and business interests in beef, buffalo, and pork are enormous, and there should be a trustworthy but low- cost method for their discrimination in the food chain.

1.3 Problem Statements

Morphological, protein and lipid based methods for animal material authentication are not reliable because of the breakdown of the analyte biomarkers during food processing. Consequently, DNA based PCR methods with short-length target have been evolved as the method of choice. Currently, three PCR-based analytical tests have been documented for simultaneous identification of beef and buffalo (Rea, Chikuni, Branciari, Sangamayya, Ranucci, & Avellini, 2001; Gupta, Rank, & Joshi, 2012; Vaithiyanathan & Kulkarni, 2016). Rea et al. (2001) and Gupta et al. (2011) developed a duplex PCR assay wherein they targeted 113- and 152-bp sequences for the beef and buffalo, respectively,

in the cytb gene and, recently, Vaithiyanathan et al. (2016) targeted 126 and 226-bp sites in the D-loop gene. However, all the reported assays have just one target region for bovine and buffalo. Moreover, there is no PCR assay that can detect bovine, buffalo and porcine materials in a single assay platform. Furthermore, they are not validated for process food analyses. Compared with a single target assay, two target assay involving two different regions for each species would be especially advantageous, since it would provide greater certainty since it is unlikely that both targets would be lost during processing. Therefore, development of mPCR assay involving two different gene sequences involving short-length amplicons for the identification of bovine, buffalo and porcine materials in foods would be greatly advantageous.

1.4 Research Objectives

1.4.1 General Objective

The overall aim of the study was to develop and validate a multiplex PCR assay targeting two different genes for each of bovine, buffalo and porcine species for the authentication of their ingredients in processed food.

1.4.2 Specific Objectives

i) To develop primers and probes targeting double genes sites for the each of bovine, buffalo and porcine species.

ii) To develop and validate a multiplex conventional and real-time PCR systems for the sensitive and reliable detection of bovine, buffalo and porcine species under various food processing treatments and complex matrices.

iii) To assess the assay performance for the screening of bovine, buffalo and porcine in processed meat products available in markets.

1.5 Scopes of Research

1.5.1 Development of Biomarkers

Nowadays, in addition to food authentication, DNA based techniques are applied for the molecular identification of pathogens in agriculture, environmental monitoring, bio diagnostics, bio terrorism and forensic analysis (Rahman, 2015a). Recently, researchers have paid more attention to the short amplicon length biomarkers due to their extra-ordinary stability against severe food processing treatments since they still can traceable in the specimen which has been treated with high pressure and temperature (Ali et al., 2012a). Previous literatures recorded that longer target DNA is susceptible to break down under harsh processing treatment causing there is a chance of false negative results (Ali et al., 2016). Although, longer amplicons are detectable but it has been proved that the shorter amplicons (≥150 bp) are more sensitive than longer ones (Ali et al., 2015c; Rojas & Isabel Gonza´ lez, 2010). Due to the extensive sensitivity and stability of the shorter amplicon DNA target, it has vast application in forensic analysis, biochip and biosensor development.

Multiplex polymerase chain reaction (mPCR) is a greatly useful molecular biology technique by which multiple targets can amplify simultaneously from a single reaction mixture. They also reduce both analytical time and cost. In this regard, multiplex mPCR assay, especially the double gene targeting one with short amplicon targets, would be especially useful and trustworthy for the simultaneous detection of species in various food products. Because of the presence of more than one target for the same species, the detection of the missing target would be complemented by a second target because it is highly unlikely that both targets would be broken down under the state of decomposition. To address this issue, I have designed a total of six sets primers with amplicon sizes of 73-146 bp, one set from mitochondrial cytochrome b (cytb) and one set from NADH dehydrogenase subunit 5 (ND5) gene of each (cow, buffalo and pig) species i.e. two sets

from cow, two sets from buffalo and two sets from pigs. The mitochondrial DNAs (mt-DNA) are more focused over the nuclear ones (n-DNA) for authentication studies because of its maternal origins, extra protection by mt-membrane and abundance in multiple copies. Hence, cytb and ND5 genes were targeted because of their higher degree of divergence and availability of sufficient conserved regions within the species but adequate polymorphism among the closely related species (Mohamad et al., 2013). Thus, the proposed activities will develop a double gene and short amplicon targeting mPCR assay for the discriminatory authentication of bovine, buffalo, and porcine materials in the food chain.

1.5.2 Evaluation of the Biomarker-specificity using PCR-based Technique

It is very important to evaluate the specificity of the developed biomarkers by using a well-known system to avoid ambiguity. The performance of the developed biomarkers of three target species (cow, buffalo and pig) were analyzed using PCR techniques. PCR is a powerful and authentic biochemical tool for the species identification in food products (Ali et al., 2011). It is an in vitro amplification processes in which specific oligonucleotide primers hybridize to the complementary target region of the DNA template followed by the enzymatic reactions of Taq DNA polymerase were occurred to complete the process (Rahman, 2015a). The amplified specific products are separated and visualized by using agarose gel under a gel image documentation system or automated capillary electrophoresis system to get better resolution. Endpoint PCR systems are not provided enough information to verify and authenticate the PCR products. Thus, sequencing of the PCR products coupled with restriction fragment length polymorphism (RFLP) was used to authenticate the amplified products if the amplicon contains appropriate restriction site (Rashid, 2015a). However, end point PCR assay is only qualitative detection scheme and unable to provide quantitative information such as amount of adulterant present in the specimen. In contrast, real-time multiplex PCR assay is a suitable tool for the

identification, differentiation and quantification of many different target species using TaqMan probe containing fluorescent reporter dye (Molenkamp, van der Ham, Schinkel, & Beld, 2007). Therefore, this research proposed the performance of the developed oligonucleotide biomarkers with simplex and multiplex conventional PCR, PCR-RFLP and TaqMan probe real-time PCR assay for detection, differentiation and quantification analysis of bovine, buffalo and porcine DNA in the food chain.

1.5.3 Assay Validation and Food Analysis

To check the validity of the developed authentication tool is a vital step because the reliability of the assay depends on the validity performance. For example, protein based methods are not suitable for the analysis of extensive processing, food due to their lack of stability and specificity (Lago et al., 2011). Hence, initial performance of the developed multiplex system was tested using the extracted DNA under raw state of target species and some other non-target species which were commonly used in meat products. Secondly, the assay was validated under different cooking conditions, namely, boiling, autoclaving and microwave cooking to realize the stability of the developed multiplex system. Subsequently, the assay sensitivity and specificity were evaluated by testing under binary and ternary admixture of target meats analysis. Adulteration as well as fraudulent labeling in the meat products is an emerging and sensitive issue. However, to identify the origin of meat in the food chain has been a concern for the protection of consumer right, public health, religious believe etc. (Arslan, Ilhak, & Calicioglu, 2006). Therefore, it is a universal desire that does not substitute the high valued declared species, entirely or partially with other lower value ones (Mafra, Ferreira, & Oliveira, 2007). Beef, buffalo and pork are the major meat of economic, religious and health concern. Although, a vast majority of the world's populations prefers beef to buffalo, but beef is totally forbidden for Hindus. Beef is also avoided in some places because of the scary of bovine spongiform encephalopathy or mad cow disease (Dalmasso et al., 2004). On the other

hand, pork is a popular meat in most of the western countries in spite of its total unacceptability to the Muslims and Jewish consumers (Ali et al., 2012a). Furthermore, the sensitivity and efficiency of the PCR assay often reduce in case of food and meat products due to the presence of various spices and additives which act as inhibitor for the binding of primers at specific site (Bottero, Civera, Anastasio, Turi, & Rosati, 2002; Calvo, Zaragoza, & Osta, 2001b; Di Pinto, Forte, Conversano, & Tantillo, 2005). Therefore, finally, the developed assay was validated under various laboratory made model and commercial food matrices such as burger, meatball and frankfurter which are popular and available. Thus, the novel assay demonstrated sufficient merits to be used by regulatory bodies for beef, buffalo, and pork authentication even in degraded specimens.

1.6 Thesis Organization

This thesis comprises of six chapters namely introduction, literature review, materials and methods, results, discussion and conclusion and future recommendations. The contents of each chapter are described below:

Chapter 1 (Introduction): This chapter described briefly the background of the study, project rational, problem statement, objectives and scope of the present research. I described here, importance of the present research, with a short description of the drawback of the previous work and also explained the innovation of the present method to overcome the limitation of the previous reports.

Chapter 2 (Literature Review): This chapter consist of detailed literature review on importance of food authentication, prevalence and impact of food fraud, importance of bovine, buffalo and porcine detection, current species identification techniques and validation of PCR methods.

Chapter 3 (Materials and Methods): All materials and protocols as well as bioinformatics tools used in this study were described in this chapter.

Chapter 4 (Results): Outcome of the experiments were illustrated here, these include extraction of DNA, designed of biomarkers, specificity of biomarkers, sensitivity and validity of the assay in various matrices and PCR products authentication.

Chapter 5 (Discussion): The experimental findings and outcomes were elaborately discussed and compared with previous reports.

Chapter 6 (Conclusion and Recommendation): Finally, findings summary of the present study including remarks and suggestion of future research were presented here.

CHAPTER 2: LITERATURE REVIEW

2.1 Influence of Animal Materials in Food Chains and Needs of Authentication

Definitive identification and quantification of animal materials have enormous interest in food, pharmaceutical and personal care products. Authentication of animal materials limits the spread of zoonotic threats, prevents unfair competition in business settings, boosts up consumer confidence and product sales, and brings long term benefits in public health, social harmony, economic growth and biological conservation of endangered species (Goffaux et al., 2005; Kitpipit, Sittichan, & Thanakiatkrai, 2014). In this regard, public awareness, regulatory laws and authentication tools work side by side to achieve these overall objectives.

Meat and meat products are widely consumed worldwide as a source of high quality protein, essential amino acids, vitamins and necessary minerals (Bai et al., 2009; Bender, 1992). According to Pimentel & Pimentel (2003) approximately two billion people globally depend primarily on meat-based diet to meet their protein demands. The Organization for Economic Cooperation and Development (OECD) and Food and Agriculture Organization (FAO) data sources reflected that in 2015 global livestock and meat consumption was roughly 198 million kg (OECD/FAO, 2016). Thus the huge markets and opportunities of meat and meat products have made them especially susceptible to adulteration and fraud labeling (Ali et al., 2014c). Survey reports of various markets reflect that the practice is going on in rampant all over the world. For example, 68% of meat products in South Africa (Cawthorn, Steinman, & Hoffman, 2013), 19.4% in the USA (Hsieh, Woodward, & Ho, 1995), 33% in the Gulf countries (Bourguiba-Hachemi & Fathallah, 2016), 22% in Turkey (Ayaz et al., 2006) and 8% in the UK (Ali et al., 2014c) were found to be mislabeled; especially beef and mutton were frequently substituted by pork, buffalo and horse meat in various instances (Ali et al., 2015c;

Cawthorn et al., 2013). It has been posing a great threats to public health because some animal species are the potential careers of multiple infecting agents that can infect humans and bring a regional emergency (Ali et al., 2014c). The US department of Agriculture (USDA) alerted that approximately 75% of human infections may come from animal products either by direct or indirect contact in different routes (USDA, 2015).

Animal materials and meat scandals can also provoke social unrest and take innocent lives. For example, beef is prohibited for the Hindus and pork is forbidden for the Muslims, Jewish and selective denominations Christians. Therefore, the indiscriminate uses of these materials are very sensitive religious and social issues and might destroy social harmony. For example, a man was killed and his family members were seriously beaten in the city of Dadri in India by a group of radical Hindus based on a false rumor that beef was kept in his refrigerator is a strong piece of evidence that meat scandal can provoke social unrest and take innocent lives (Matthew, 2015). Therefore, it is a vital need to assure that meat and meat products are not adulterated and properly packaged, labeled and marketed for the safeguard of public health, religious faiths and of course fair-trade economic practices in food businesses (Kitpipit et al., 2014).

2.1.1 What We Mean by Adulteration

According to Food and Drug Administration (FDA), adulteration is the replacement of higher valued ingredients by cheaper ones for the purpose of economic gain. Thus food adulteration is defined as a deliberate act of degrading the quality of food products by fraudulent admixing or substituting lower-grade ingredients for its highest valued counterparts for financial gain or additional profit (Rahman, 2015a).

According to Federal Food, Drug, and Cosmetic (FD & C) Act (2002, Sec. 402) of the United States (Adulterated Food, 2002; Rahman, 2015a), a food shall be deemed to be adulterated:

a) "If it bears or contains any poisonous or deleterious substance which may render it injurious to health.

b) If it bears or contains a pesticide chemical residue, food additive, or a new animal drug (or conversion product thereof) that is unsafe for public health.

c) If it consists in whole or in part of any filthy, putrid, or decomposed substance, or if it is otherwise unfit for food.

d) If it has been prepared, packed, or held under insanitary conditions whereby it may have become contaminated with filth, or whereby it may have been rendered injurious to health.

e) If it is, in whole or in part, the product of a diseased animal or of an animal which has died otherwise than by slaughter.

f) If its container is composed, in whole or in part, of any poisonous or deleterious substance which may render the contents injurious to health.

g) If it has been intentionally subjected to radiation, unless the use of the radiation was in conformity with a regulation or exemption in effect.

h) If any valuable constituent has been in whole or in part omitted or abstracted therefrom.

i) If any substance has been substituted wholly or in part therefore.

j) If damage or inferiority has been concealed in any manner.

k) If any substance has been added thereto or mixed or packed therewith so as to increase its bulk or weight, or reduce its quality or strength, or make it appear better or of greater value than it is".

On the other hand, the European Union (EU) has not clearly defined the term of food fraud or food adulteration, but the term was recognized as an intentional action that occurred for the purpose of monetary profit. The aim of the EU food law include to prevent (i) deceptive or fraudulent practices; (ii) food adulteration and (iii) any other activities which may cheat the consumers (Avery, 2014; Rahman, 2015a). The EU

introduced the food law regarding food safety to protect consumers' health. The EU requirements of the food safety are as follows:

"i) Food shall not be placed on the market if it is unsafe.

ii) Food shall be deemed to be unsafe if it is considered to be: (a) injurious to health; (b) unfit for human consumption.

iii) In determining whether any food is unsafe, the following criteria should be addressed: (a) if the normal conditions of the food consumed by people and the safety is controlled at each stage of production, processing and distribution, and (b) if necessary information is provided to the consumer, including the general information on the label, or other information concerning the avoidance of specific adverse health effects from a particular food or category of foods.

iv) In defining whether any food is injurious to health, regard shall be had: (a) not only to the probable immediate and/or short-term and/or long- term effects of that food on the health of a person consuming it, but also on subsequent generations; (b) to the probable cumulative toxic effects; (c) to the particular health sensitivities of a specific category of consumers where the food is intended for that category of consumers.

v) In determining whether any food is unfit for human consumption, regard shall be had to whether the food is unacceptable for human consumption according to its intended use, for reasons of contamination, whether by extraneous matter or otherwise, or through putrefaction, deterioration or decay.

vi) Where any food which is unsafe is part of a batch, lot or consignment of food of the same class or description, it shall be presumed that all the food in that batch, lot or consignment is also unsafe, unless following a detailed assessment there is no evidence that the rest of the batch, lot or consignment is unsafe.

vii) Food that complies with specific Community provisions governing food safety shall be deemed to be safe insofar as the aspects covered by the specific Community provisions are concerned".

The government of Malaysia established the Department of Standards Malaysia whose aims include the protection of consumers' health and safety by assuring the standard of the manufacturing and trade of halal food (MS, 2009; Rahman, 2015b). According to the Department of Standards Malaysia food and drink and/or their ingredients permitted under the *Shariah* law must fulfill the following criteria:

"a) It does not contain any parts or products of animals that are non-halal by *Shariah* law or any parts or products of animals which are not slaughtered according to *Shariah* law;

b) It does not contain najs (dogs and pigs and their descendents/non-halal contaminants) according to *Shariah* law;

c) Food should be safe for consumption, non-poisonous, non-intoxicating or non-hazardous to health;

d) Food not prepared, processed or manufactured using equipment contaminated with *najs* according to *Shariah* law;

e) Food does not contain any human parts or its derivatives that are not permitted by *Shariah* law;

f) During its preparation, processing, handling, packaging, storage and distribution, the food items a), b), c), d) or e) or any other things that have been decreed as *najs* by *Shariah* law".

2.1.2 Mislabeled Food

Food fraud practice is not a new innovation but it has been started since the Roman and Greek Empires, there were rules concerning the adulteration of wines with colors and flavors (Charlebois, Schwab, Henn, & Huck, 2016; Shears, 2010).

A food control regulation was established in Germany and France in 13th century. At the same time, King John prepared a circulation of penalties for bread adulteration in England (Shears, 2010). However, deceptive mislabeling of food products, especially meat and meat products, particularly the expensive one, has recently becomes a widespread issue (Rojas, González, García, Hernández, & Martín, 2012). For example, according to Agriculture's Food Safety and Inspection Service (FSIS) about 12,566 pounds of pork, beef, and poultry products were recalled due to mislabeled in 2015 (FSW, 2015). In 2015, another mislabeled scandal, imported and farm raised about 25,000 pounds of shrimp was sold as wild caught product (FSN, 2015). Moreover, Chuah et al., (2016) found 78.3% of tested samples were mislabeled in Malaysia.

These have increased consumer's concern about the composition and origin of food products, particularly in meat and meat products (Rojas et al., 2011). Appropriate product labeling with proper description is very conclusive for consumers because it respects personal food choice, safeguards the public health, assures fair trade and religious belief (Ali et al., 2015d). The authenticity of the finished food product's depends on their compliance with labeling rules and regulations, mainly in terms of the composition of ingredients, manufacturing methods and practices, genetic identity and technology (Charlebois et al., 2016).

Under the Federal Food, Drug, and Cosmetic Act of United State section 403 (MBF, 2002) stated that a food considered as misbranded or mislabeled-

" a) If its labeling is false or misleading in any particular, or its advertising is false or misleading in a material.

b) If it is offered for sale under the name of another food.

c) If it is an imitation of another food, unless its label bears, in type of uniform size and prominence, the word ''imitation'' and, immediately thereafter, the name of the food imitated.

d) If its container is so made, formed, or filled as to be misleading.

e) If in package form unless it a label containing (1) the name and place of business of the manufacturer, packaging provider, or distributor; and (2) an accurate statement of the quantity of the contents in terms of weight, measure, or numerical count, except that under clause (2) of this paragraph reasonable variations shall be permitted, and exemptions as to small packages shall be established, by regulations prescribed by the Secretary.

f) If any word, statement, or other information required by or under authority of this Act to appear on the label or labeling is not prominently placed thereon with such conspicuousness (as compared with other words, statements, designs, or devices, in the labeling) and in such terms as to render it likely to be read and understood by the ordinary individual under customary conditions of purchase and use.

g) If it purports to be or is represented as a food for which a definition and standard of identity has been prescribed by regulations as provided by section 401, unless (1) it conforms to such definition and standard, and (2) its label bears the name of the food specified in the definition and standard, and, insofar as may be required by such regulations, the common names of optional ingredients (other than spices, flavoring, and coloring) present in such food".

2.1.3 Prevalence of Meat Food Fraud

The demand for meat and meat products are rapidly increasing with the increasing world's population. Unfortunately, despite having national and international rules and regulation in most of the countries (Kitpipit et al., 2014), adulteration of meat and meat products is going on in rampant; this is just to make extra profit and outweigh the honest companies in the competitive markets (Ali et al., 2012a; Hou et al., 2015). The recently made grouper (*Epinephelus marginatus*) meals authentication studies in Madrid restaurant reflect that only 9 out of 37 samples contained authentic species (Asensio, 2008) and 22% meat products 22% in Turkey were mislabeled (Ayaz et al., 2006), 19.4% in the USA (Hsieh, Woodward, & Ho, 1995). Similarly, false or wrong labeling were found in about 8% meat products in the United Kingdom and 15% in Switzerland (Ali et al., 2014c). Fraud labelling was also found in the deer products, particularly blood, heart and antler products as elucidated by Zha, Xing, & Yang (2010). Ulca, Balta, Çağın, & Senyuva, (2013) demonstrated that chicken and turkey were found instead of beef in 100% beef labeled meatballs and no bovine DNA was found in sausages labeled as 5% beef in Turkey. Verification of beef and pasta products in the UK showed that 29 out of 2501 samples contained 1% horse DNA (Castle, 2013). The Food Safety Authority of Ireland also detected horse DNA in 37% of the tested beef burgers and 85% of them also contained pig DNA (Walker, Burns, & Burns, 2013). Cawthorn et al. (2013) found that 68% (95 of 139) samples of burger patties, sausages and deli meats contained species which were not indicated on the product labeling. Pig DNA was detected in 30% of burger and patties, 52% of sausages, 32% of deli meats and 38% of minced meat products as undeclared species. Al-Nassir et al. (2014) identified undeclared species in 24% of beef burgers and minced meat samples. A total of 105 imported beef products were analyzed by Bourguiba-Hachemi et al. (2016) in the Arabian Gulf regions, and they found positive results for pig and horse species in 26% and 7% of the tested samples. Recently, police

seized over 20 tons of fake beef which was made up from chemically treated pork in Shaanxi province of China (Tan, 2013). In another incident, Chinese police arrested 904 suspects who were involved in the selling of processed rat meat as lamb (Buckley, 2013). In the recent years, Malaysia also faced some challenges such as porcine DNA in Cadbury chocolate (Rahman et al., 2015b), lard in bread and pig intestine casings in sausages (Man, Aida, Raha, & Son, 2007). Surprisingly, Clear Labs identified human and rat DNA in burger samples in northern California (Kowitt, 2016). The Clear Labs also found porcine DNA in beef burgers and beef DNA was found in ground lamb and pathogens DNA was found in 4.3% of tested food products (Kowitt, 2016; Labs, 2016). The above incidents are just some of the many phenomena of animal product adulterations that are taking place all over the world but sufficient to demonstrate that food products should be authenticated for their animal origins to promote fair-trade economic practices and prevent fraudsters from harming our public health, religious faith and personal budgets.

2.1.4 Impact of Food Fraud

Deceiving consumers by selling fraud foods is not a current issue. It not only causes an economic loss but also may put consumer on serious health risk because some people are allergic to certain food ingredients. In the 18^{th} and early 19^{th} centuries, numerous poisonous substances were used as food additives, for examples, chalk and alum were added as an whitening agent in bread; and sawdust, pipe clay or calcium sulfate was used to increase the volume or the weight of the bread (Tähkäpää, Maijala, Korkeala, & Nevas, 2015). During that time, lead was mixed with beer and wine; and sand, dirt and other leaves were regularly added to tea, coffee and spices (Schumm, 2014). In 1902, Dr. Harvey W. Wiley and co-workers who are known as the Poison Squad showed that food preservatives which were used at that time such as copper sulfate, sulfuric acid, borax and formaldehyde have the adverse effect in the body (Schumm, 2014). A remarkable incidence involving toxic oil syndrome that took 300 initial deaths and finally a total of

1663 deaths out of 20,000 affected people in Spain in 1981 due to the consumption of industrial oil as olive oil (Borda et al., 1998; Gelpí et al., 2002). Another thunder like fiasco was the Chinese milk and baby formula adulteration with melamine in 2008 (Guan et al., 2009). After ingesting the melamine contaminated infant formula and milk, approximately 300,000 infants and children were affected with urinary tract stones and at least six were killed (Reshanov, 2008). In 1986, 23 persons were died due to methanol contamination with wine in Italy (Tähkäpää et al., 2015).

Although meat and meat products forgery is not so much detrimental to health, it is a very sensitive religious and cultural issues that might provoke social unrest and extirpate certain endangered species from the world's natural habitats. Meat wholesaler of Japan mislabeled imported beef as domestic beef during the government buyback program after the bovine spongiform encephalopathy (BSE) crisis (Yeboah & Maynard, 2004). In 2005, Sudan (non-permitted color) was found in some meat products in China (Jia & Jukes, 2013). In 2003, inedible poultry meat of pet food plant got entry into the food chain in the UK (FSA, 2004). A severe food crisis was exposed in Belgium due to contamination of cancer-causing dioxin and polychlorinated biphenyls (PCBs) in 1999 (Covaci et al., 2008). The crisis happened due to the contamination of fat used for the production of animal feed with 1 gm dioxins and 50 kg PCBs; this resulted in adverse effect on domestic and export market of poultry and pork because about 2500 poultry and pig farms were affected (Buzby & Chandran, 2003; Covaci et al., 2008) and the USA cancelled the import of certain types of food products from the entire European Union (Kennedy, Delaney, McGloin, & Wall, 2009). In Ireland, the Irish pork dioxin crisis in 2008 affected pork market because approximately 10% pig was affected due to the feeding of dioxin contaminated feed. Consequently, all pork products which were manufactured during this time were recalled, causing huge loses to the manufacturing industries (Kennedy et al., 2009). Poultry and beef of unknown sources were repackaged and marketed illegally as

human food in Northern Ireland (Tähkäpää et al., 2015); in 2007 poultry were diseased and blanched, was marketed for human consumption in the UK (North, 2013; Rahman, 2015a) and in 2006, approximately 150 tons of spoilt meat was distributed Germany (Anonym, 2007). These incidences reflect that adulteration and/or mislabeling of food was a common issue worldwide for many years ago to till date and conceivably a never-ending event. Consumers are not only the victim of food forgery, but also the government and even some time businessmen are greatly affected (Rahman, 2015a). Regarding the social and cultural issues, Mr. Mohammad Akhlakh was killed and his family members were beaten severely by a radical Hindu mob in Dadri in India because rumor was spread that the family had consumed beef and also stored in the refrigerator for future uses (Matthew, 2015). Thus, authentication of food ingredients bear huge influences to safeguard our public health, food choice and preferences and of course religion compliances (Ali et al., 2014a).

2.2 Importance of Cow, Buffalo and Pig Species Detection

2.2.1 Meat Sources

Meat and meat products are an important part of the human diet because they contain proteins and essential amino acids as well as minerals, vitamins and micronutrients which are very essential for the development, growth and good activities of the body organisms (FAO, 2014; Pimentel & Pimentel, 2003). Especially, high nutrient containing foods such as meat and meat products are essential for children and women as well as the communities infected with HIV or AIDS (FAO, 2014). Consequently, many countries consider the meat-based diet as one of the basic and significant nutritional food and establishing an important part of the food manufacturing industry. Thus, approximately 2 billion populations depend primarily on meat-based food worldwide (Pimentel & Pimentel, 2003). The common meat source is livestock species such as cow, pig, buffalo, goat, sheep and poultry (Herrero, Royo, Lago, Vieites, & Espiñeira, 2013). Among the

livestock species, cow, buffalo and pig are the popular and a major source of meat and meat products regarding the cultural, lifestyle, religious and geographical preferences. Global production and consumption of meat increasing dramatically day by day commensurating the linear rising in the global human population (Wanapat & Chanthakhoun, 2015). According to Worldwatch Institute, production of meat has increased 3 times during the last four decades and it has been risen 20% in the last decade (Rousseau, 2016; WWI, 2017). Moreover, Todd Reubold assumed that consumption of global meat will be increased more than 4% per capita over the next 10 years (Reubold, 2015). FAO reported that annual meat consumption increased from 10 kg to 26 kg between 1960 and 2000 and it has been projected that it will increase further to 37 kg over the year 2030 (Wanapat & Chanthakhoun, 2015). The figure of meat consumption per capita per year in different countries as projected by the Organization for Economic Cooperation and Development (OECD) is given in Figure 2.1 (OECD, 2017b).

A list of highest meat consuming countries with the total amount (Kg) of meat (beef and veal, pork, sheep and poultry) consumed per capita in 2015 was calculated and illustrated in Figure 2.2 from the OECD data (OECD, 2017b).

The Figure 2.2 clearly shows that the United States is the highest meat consuming country wherein each person eats about 95.4 kg of meat per year or nearly 260 gm per day. Whereas each resident in South Africa consume 47.8 kg of meat per year or about 130 gm a day as the lowest meat eating country. The global average meat consumption of an adult in 2014 was 34 Kg and it will increase to 35.5 kg by 2024. Therefore, global consumption of meat will increase more than 4% per capita over the next decade (Reubold, 2015). To fulfil the global annual meat demand, the huge numbers of livestocks and poultry are required, such as 1.5 billion cows, 1 billion sheep, 1 billion pigs and 19 billion chickens, it is more than three times the number of the world population (Reubold,

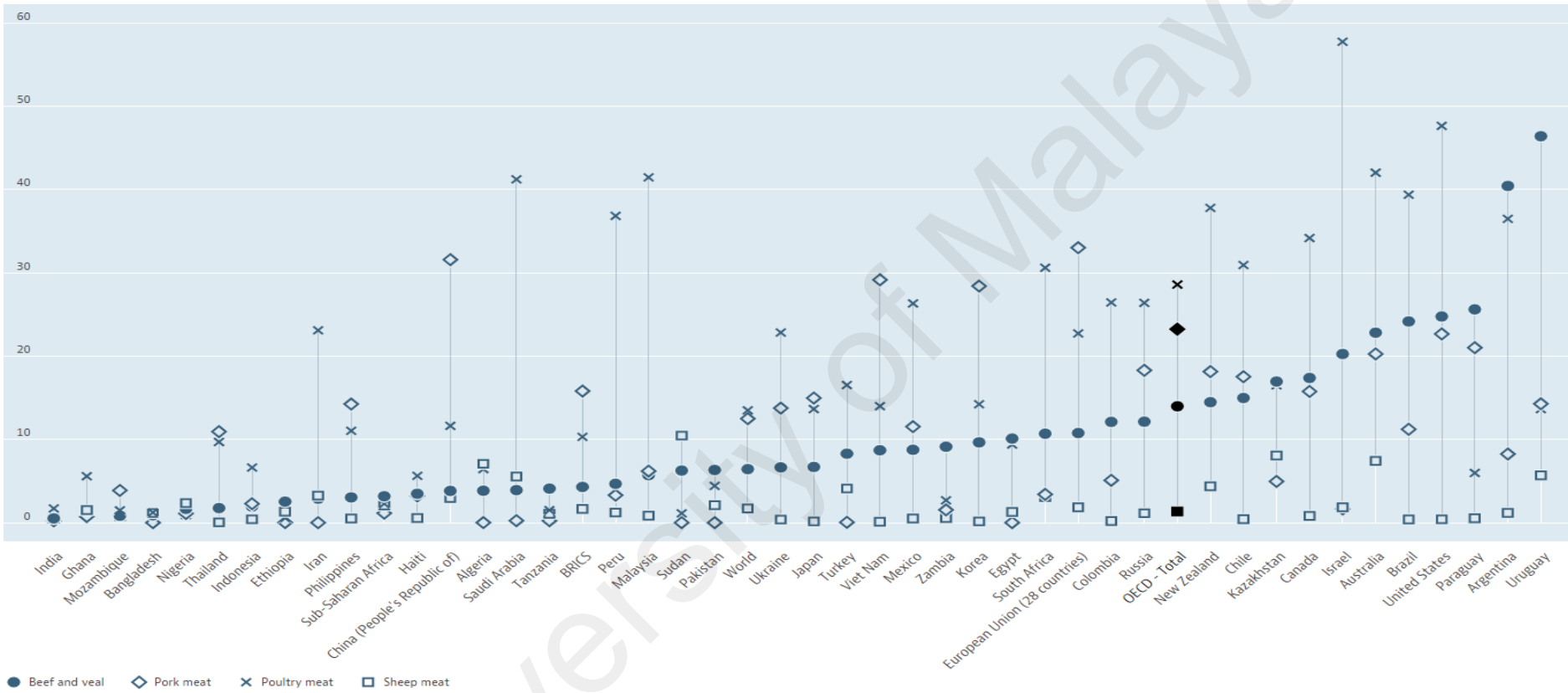

Figure 2.1: Consumption of meat (beef and veal, pork, lamb and poultry) by different countries in 2015 as projected by OECD. (data source: OECD, 2017b)

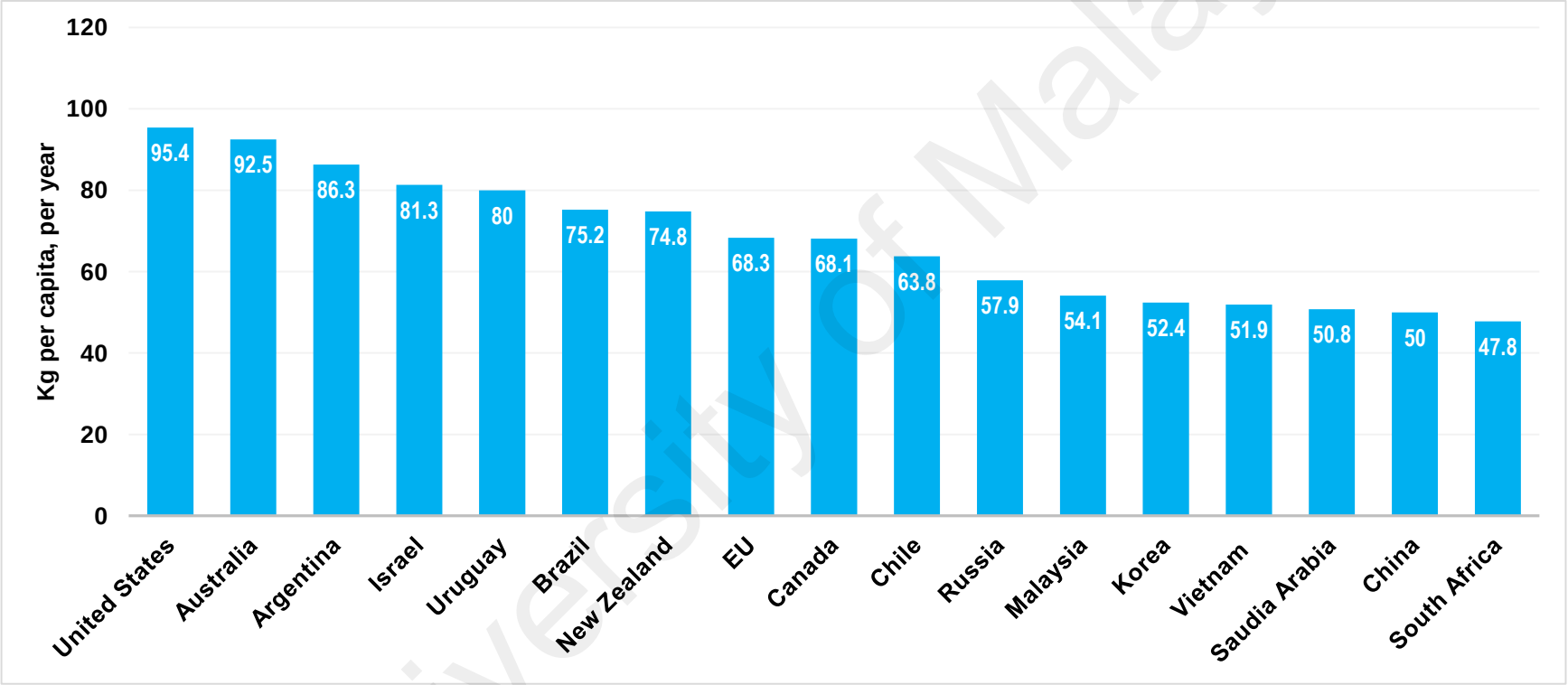

Figure 2.2: List of top meat (beef and veal, pork, sheep and poultry) consuming countries in 2015 (Kg per capita, per year) (data source: OECD, 2017b)

2015). According to the FAO, 25 million of cows, buffalo, goats and sheep are increasing annually (Reubold, 2015) and to meet the yearly meat consumption demand, about 9 billion livestock are required only in the United States and it is about 5 times more than the US people (Pimentel & Pimentel, 2003).

Livestock animals also have a major impact on the environment as said by the prominent ecologist, Brain Machovina that "The livestock sector is responsible for approximately 15 percent of all human greenhouse gas emissions which is equivalent to all the direct emissions from transportation" (Reubold, 2015). In 2014, the five highest beef consumed per capita were Argentina, Uruguay, Brazil, the United States and Australia. It has been predicted that five more countries such as Vietnam, Indonesia, Mozambique, Turkey and Tanzania will be included in the list of top beef consumption countries between 2014 and 2024 (Reubold, 2015). According to an EU livestock survey, cows represent 18.8% of total animal output and 8.1% of agricultural yield. Production of beef in the EU has increased by 7% for young cattle and calves and 3% for adult cows from 2009 and 2014 (Marquer, Rabade, & Forti, 2015). Whereas pork denoted 9.0% of the total EU agricultural output .

The scenario of the total consumption of livestock meat and poultry in 2015 was represented by Pork checkoff from the USDA Foreign Agricultural Service and UN Food and Agricultural organization data source (Figure 2.3). According to the pie chart, the highest proportion of consuming meat was pork with 40.1% of the total and the second largest consumption was a poultry accounted for 34.1%, followed by beef with 21% in 2015 (Anonym, 2016b).

From the report of FAO 2008, the estimated global buffalo population is approximately 177 million and 97% (171 million) of that are found in Asia, while the rest of the buffaloes (3%) are found in other countries (Wanapat & Chanthakhoun, 2015).

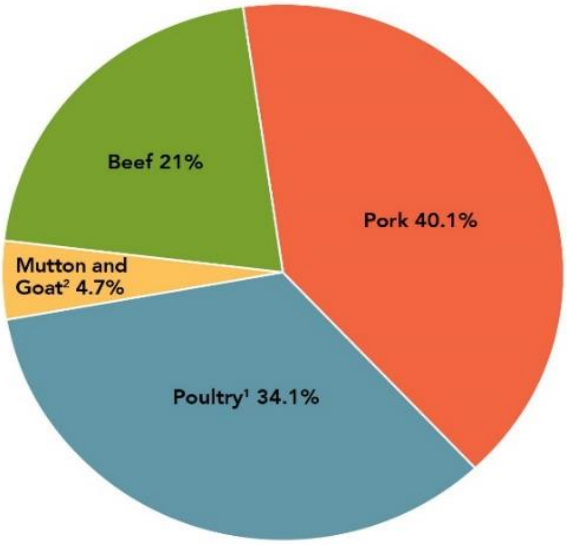

Figure 2.3: World meat and poultry consumption shares in 2015. Sources: USDA Foreign Agricultural Service, UN Food and Agricultural Organization. [1]Includes turkey consumption for 2014, the most recent year for which data are available, [2]Includes mutton/goat consumption for 2011, the most recent year for which data are available (Anonym, 2016b).

In Asia, only 21.0% of the total meat production comes from livestock species and buffalo meat constitutes approximately 11.52% of the total livestock meat (Cruz, 2010). The consumers did not like buffalo meat in the past and so there was very little trading in the past century, but now buffalo meat consumption has been greatly increasing day by day. This is because buffalo meat contains significant attribute such as low cholesterol, fat (below 2%) and calories but higher in iron and essential amino acids as well as free from Mad Cow Disease (Badpa & Ahmad, 2014). As a result, the global buffalo population has increased annually by about 1.49% over the last 10 years (Badpa & Ahmad, 2014). Similarly buffalo meat marketing is also growing up significantly. The highest buffalo meat exporter countries are India, Brazil and Australia, but India is the largest exporter among these countries. According to USDA outweigh 2.4 million tons buffalo meat were exported from India, where as Brazil and Australia exported 2.0 and 1.5 million tons, respectively in 2015 (Wikipedia, 2016). Since 2011, export of buffalo meat from India is

increasing yearly on average approximately 14% and India earned more than 4.8 million US dollars in 2014 (Cruz, 2010).

2.2.2 Domesticated Animals as a Source of Meat

Cow, buffalo and pig are among the five major domestic livestock animals and their production rate are increasing greatly day by day. Table 2.1 shows the comparative production of five top animal species between 2000 and 2013 (FAO, 2015).

Table 2.1: Top five livestock production in 2000 and 2013 (data source: FAO, 2015)

Species	2000 (thousand heads)	2013 (thousand heads)	Rate of Increase (%)
Cattle	1,302,895	1,494 349	14.69
Buffaloes	164,114	199,784	21.73
Pigs	856,241	977,021	14.10
Sheep	1,059,082	1,172,833	10.74
Goats	751,632	1,005,603	33.78

2.2.2.1 Cow (*Bos taurus*)

Among the domesticated animals, cows are the largest and most common. They were domesticated approximately 10,500 years ago and they belong to *Bovidae* family and *Bos* genus (Bollongino et al., 2012). Cows are basically identified as three different species, according to region:

i) *Bos taurus*, which are called "taurine" cattle or European cattle and they also include identical species from Africa and Asia.

ii) *Bos indicus*, which are called zebu and

iii) *Bos primigenius* which are known as the aurochs and currently become extinct.

However, the ancestor of both zebu and taurine cattle is the aurochs. Thus, these three groups were classified again as one species: *Bos taurus*, but divided into three subspecies such as

i) *Bos taurus taurus*

ii) *Bos taurus primigenius* and

iii) Bos taurus indicus (Wilson, & Reeder, 2005).

Domesticated cows are very important for human beings because they fulfill a major part of the human food chain by providing meat and milk and it is assumed that about half proportion of the global meat comes from cow (Rickard, & Book, 1999). Cow milk is further used for the production of many dairy products such as butter, cheese, yogurt etc. They are also used in medicine, leather, soap and glues (Dewey, 2017). According to the OECD report Uruguay is the highest beef consuming country in 2015, the amount of meat consumed per capita 46.4 kg (Figure 2.4) (OECD, 2017b). About 40.4 kg beef was consumed by an adult of Argentina since this country is the second highest in beef consumption. One person of US consumed 24.7 kg in 2015 whereas Brazil and Australia consumed 24.2 and 22.8 kg respectively.

2.2.2.2 Water Buffalo (*Bubalus bubalis*)

There are two types of water buffaloes are recognized, based on behavioural and morphological characteristic such as

i) River buffalo in Indian subcontinent and domesticated about 5,000 years ago and

ii) Swamp buffalo found in China and domesticated approximately 4,000 years ago (Yang et al., 2008).

Water buffalo belong to genus *Bubalus*, species *bubalis* and is the member of the Bovidae family. The ancestor of wild water buffalo (*Bubalus arnee*) is found to be same of the domestic water buffalo (Lau et al., 1998). The International Commission on Zoological Nomenclature concluded about naming of domestic and wild buffalo by ruling the valid in the scientific name of wild species as *Bubalus arnee,* whereas domestic buffalo is *Bubalus bubalis* in 2003 (Gentry, Clutton-Brock, & Groves, 2003).

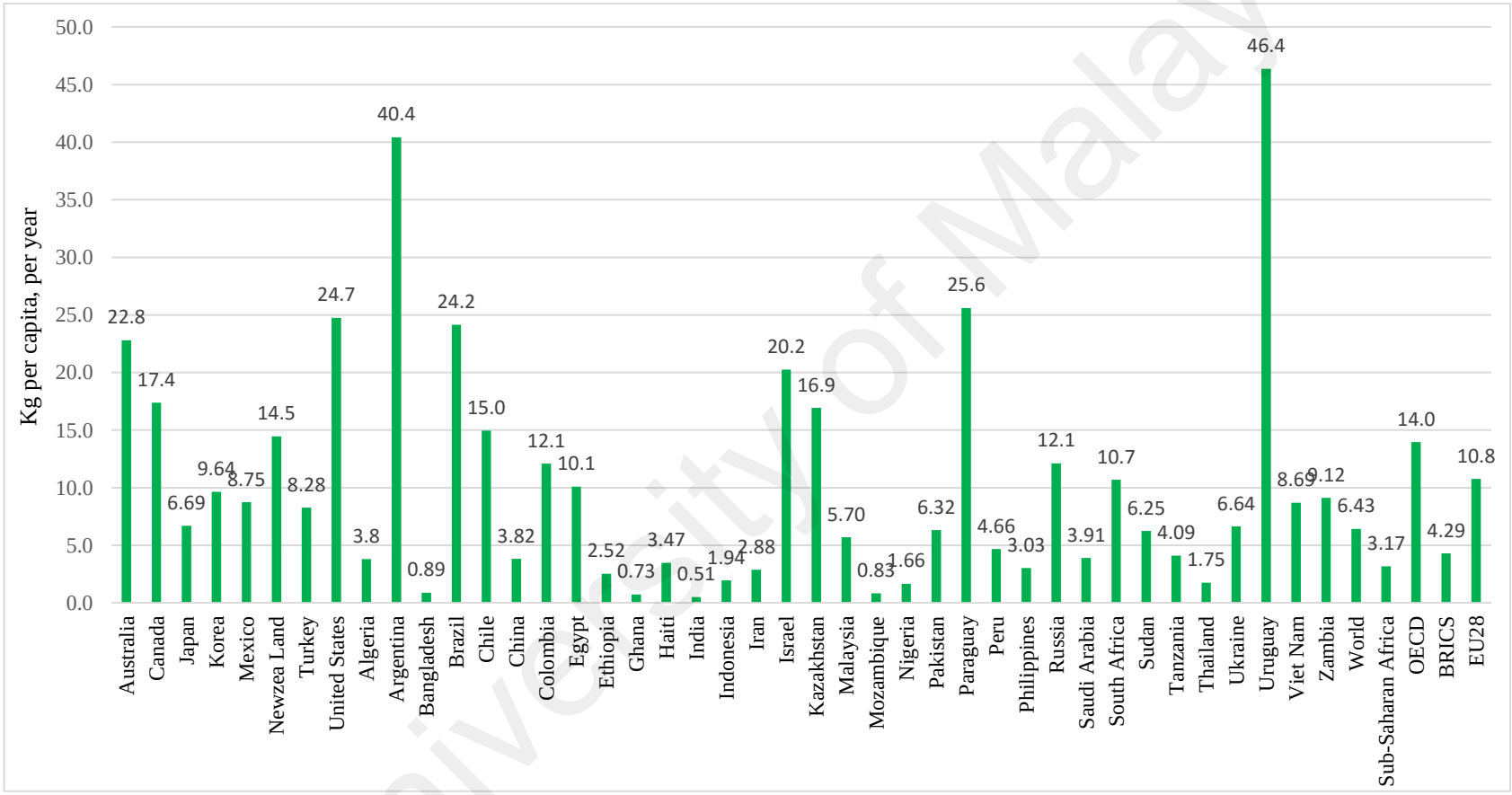

Figure 2.4: Consumption of beef by different countries in 2015 **(**data source: OECD, 2017b).

Demand of meat-based foods are increasing due to the growth of world human population, particularly in developing countries (Wanapat & Chanthakhoun, 2015). Thus, the world meat production will increase 16% in 2025 than the period 2013-15 (OECD, 2017a). Buffalo meat is considered to be a strong source for fulfilling of this increasing meat requirement (Borghese, 2005). Moreover, buffalo meat is realized as important for its local usage and potential export material in recent years. In addition, buffalo meat is known as healthful for human diet among red meats since it contains lower calories, cholesterol and intramuscular fat, higher amount of essential amino acids, mineral and biological value (Badpa & Ahmad, 2014). It is assumed that more than 150 million water buffaloes are farmed commercially worldwide today, which are farmed for meat, milk and leather production (Anonym, 2017c). India is the largest buffalo meat producing and exporting countries worldwide. Global population of buffalo is 180.7 million, among these buffalo populations in India is about 98.6 million (54.5% of total population) (Wanapat & Chanthakhoun, 2015). However, India exported 1,475,526 tons of buffalo meat in 2014-15 (Krishnakumar & Bureau, 2015).

2.2.2.3 Pig (*Sus scrofa*)

It is believed that pigs (*Sus scrofa*) were domesticated about 9,000 years ago from wild boar. They are native in Europe and parts of Asia and gradually spread to many areas of the world (Compassion, 2017). The scientific name of domestic pig is usually given as *Sus scrofa,* although some scientists call as *Sus scrofa domesticus.* Pork checkoff listed 10 countries which were produced the highest amount of pork in 2015 (Figure 2.5). In 2015, the largest pork producing country was china with the values of 54.87×10^6 metric tons, followed by European Union accounted for 23.35×10^6 and the third highest was Brazil with the amount of 11.121×10^6 metric tons. Whereas Japan was the lowest pork producing country among the top 10 countries by producing 1.254×10^6 metric tons in 2015 (Checkoff, 2016).

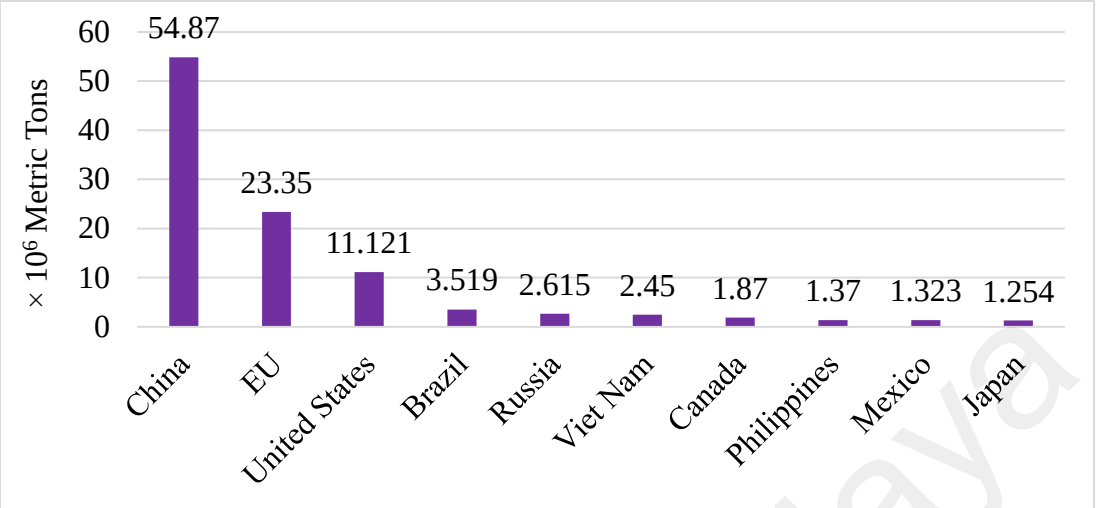

Figure 2.5: Top 10 pork-producing countries in 2015 (data source: USDA Foreign Agricultural Service) (Checkoff, 2016).

On the other hand, European Union exported the largest proportion of the global pork export making 33% of the total in 2015. The second highest exporter was the United States, which was 31% of the total, followed by 17% in Canada, 9% in Brazil, 3% in China, 2% in Chile and Mexico, 1% in other countries respectively (Figure 2.6) (Checkoff, 2016).

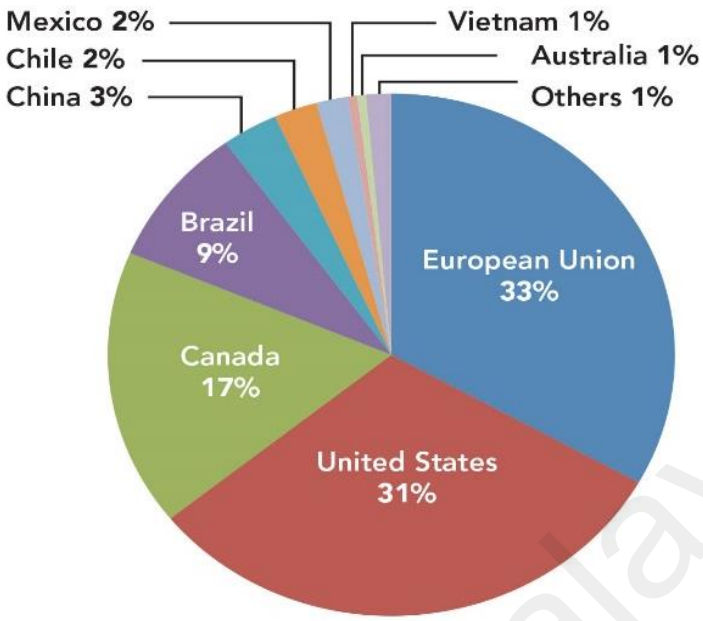

Figure 2.6: Global pork export in 2015 (Checkoff, 2016)

In case of pork consumption, OECD represented the data for the per capita consumption in 2015 (Figure 2.7). The EU was the highest pork consuming country comprised 33.0 kg per capita and the second largest pork consuming country was China accounted for 31.6 kg per capita, followed by 29.1 kg in Viet Nam, 28.4 kg in Korea, 23.2 kg in OECD countries, 22.7 kg United States, 20 kg in Australia, 18.3 kg in Russia, 18.1 kg in New Zealand and 17.5 kg in Cheli. Very little amount of pork consuming countries includes Algeria, Bangladesh, Egypt, Iran, Pakistan and Sudan as there are Islamic countries (OECD, 2017b).

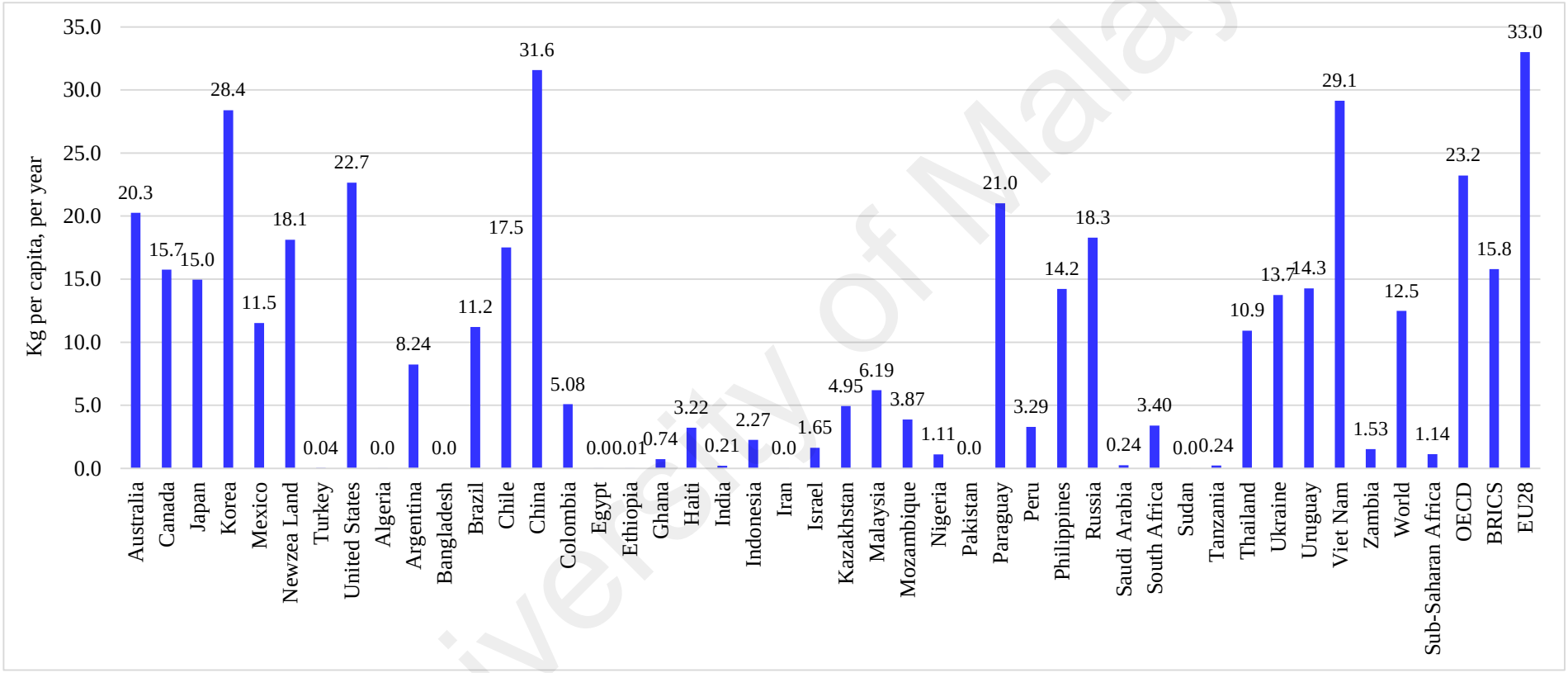

Figure 2.7: Global consumption of pork per capita in 2015 (data source: (OECD (2017), Meat consumption (indicator). doi: 10.1787/fa290fd0-en (Accessed on 06 March 2017)).

2.2.3 Religious Belief

Religious faith is also an important factor for the choice of food, particularly meat and meat products. Most religions have their own assertion for the human food consumption, specific food items are allowed to intake and others are prohibited (Meyer-Rochow, 2009). Any taboo can act as a protector and safeguard of the believers if it is considered as the supreme instruction of God for religious and cultural activities. However, beef is strictly restricted for Hindus since the cow is considered as a holy mother and very sacred animal, but there is no prohibition in consumption of milk and milk products (Meyer-Rochow, 2009). The recent incidence of killing a man and beating his family members in the city of Dadri, India by a group of radical Hindus based on a false rumor of keeping beef in his refrigerator indicates that beef is a very sensitive issue of Hinduism which can provoke social unrest and take innocent lives (Matthew, 2015). While kashrut (food law of Jewish) permits the ruminant animal species containing two criteria such as clove hooves and chew the cud. Thus, cow, goat, deer, bison and sheep are allowed, but pig, camel and rock Hyrax are forbidden for the Jews. They are prohibited to consume both the meat and the milk together (Judaism, 2016; Regenstein, Chaudry, & Regenstein, 2003). Over the past decade, the demand of kosher (food permitted to the Jews) food products has been increased drastically and currently it has been the new trend for food products (Solanki, 2016). However, kosher market is expanding, particularly in the United States and Europe as well as increasing availability of the kosher products (Jayalal, 2015; Lever, Bellacasa, Miele, & Higgin, 2010). It is calculated that approximately 40% of the shelves products in the supermarkets of the USA are kosher and 125,000 kosher products are available in US supermarkets and about 3,000 more are included in each year and the number of kosher consumers more than 45 million worldwide (Buckenhüskes, 2015; KLBD, 2017). It has been reported that over 10,000 American companies manufacture kosher products, followed by the Europe is the second highest contributor in

world kosher food market (Solanki, 2016). According to annual food sales report kosher products comprised of about 200 billion US dollars (40%) among the total food sales of 500 billion US dollars (Buckenhüskes, 2015). Mintel reported that 62% consumers buy kosher for the reason of quality food followed by 51% people for general healthfulness and 34% for food safety. Whereas only 14% consumers purchase kosher due to follower of Jewish religion (Mintel, 2009). Report on supermarket represented that growth of kosher certified products are 6%, whereas only 2% growth of non-kosher products and kosher products captured about 80% of the Israel and 21% of the US retail market (Anonym, 2017a).

The food, permitted to consume for the Muslim is defined as Halal food. The word "Halal" is an Arabic word that is referred to as what is permitted and lawful by the Shariah (Islamic Law) (Mohamed, Rahim, Ma'ram, & Ghazli, 2016). However, any food which Muslim is allowed to consume as per the description of the Quran and Hadith (the saying and practice of Prophet Muhammad (peace be upon him)) and the fiqah (interpreted by Muslim scholars) are known as Halal (Regenstein et al., 2003). The Muslim believes that Islamic Shariah allowed only the food which are beneficial for health and restricted that are harmful for the body as described in the holy book Quran (Anonym, 2017b; Halalce, 2017).

"O mankind! Eat of that which is lawful and good on the earth, and follow not the footsteps of Shaitan (Satan). Verily, he is to you an open enemy". (Quran 2.168)

"O you who believe (in the Oneness of Allah Islamic Monotheism)! Eat of the lawful things that We have provided you with, and be grateful to Allah, if it is indeed He Whom you worship". (Quran 2.172)

"He has forbidden you what dies of itself (carrion), and blood, and the flesh of swine and that over which any name other than (that of) Allah has been invoked". (Quran 2. 173)

Thus, halal animals include cows, buffaloes, goats, sheep, camel, duck, goose, chicken, ostrich, turkey etc. if they are slaughtered according to Sharia law. On the contrary, pork is one of the most common non-halal food for Muslims and also forbidden for select Christian denominations. The other non-halal foods are horse, dog, cat, alcohol etc. Recently, halal food market is expanding rapidly and is becoming a mainstream of the market due to increasing global Muslim population (Hanzaee & Ramezani, 2011). Halal foods not only the religious concern, but also becoming a global sign of quality products as well as a lifestyle choice (Hanzaee & Ramezani, 2011). It is reported that the number of global Muslim population is about 1.8 billion and that population is growing yearly at approximately 1.8% and projected to reach 36% of the global population over 2025 (Jeddah, 2011; MIHAS, 2013). According to Pew Research Center's Forum on Religion & Public Life the global Muslim population projected to reach from 1.6 billion, representing 23.4% of the global population of 6.9 billion in 2010 to 2.2 billion, representing 26.4% of the global total expected population of 8.3 billion over 2030 (Figure 2.8) (PewResearch, 2011).

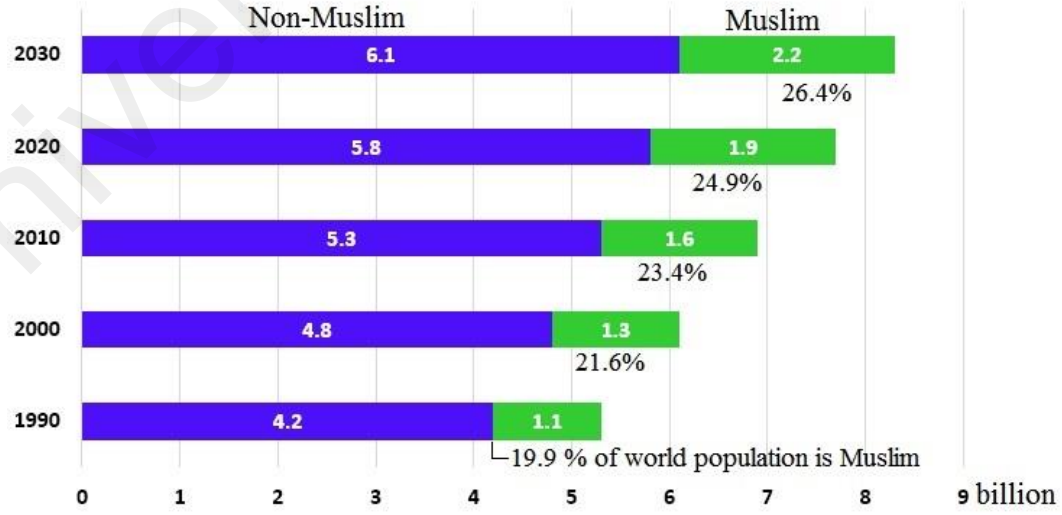

Figure 2.8: Projection of global Muslim population, 1990-2030 (Adapted from: PewResearch, 2011)

However, it is considered that halal food is one of the rapidly growing food item all over the world at present. Because halal foods comply not only halal standard, but also comply the GMP, HACCP and other recognized quality or safety standards (HAC, 2017). Thomson Reuters stated that according to Global Islamic Economy Report the growth of global Muslim consumer expenditure on food and lifestyle products increased about 9.5% from the past years and accounted for 2 trillion US dollar in 2013 and projected to reach about 3.7 trillion US dollars by 2019 with the yearly growth rate of 10.8% (Newshalal, 2016; Rasid, 2016). It was estimated that world halal food trade growth about 4.44% over 2012 to 2016 (TIBDA, 2016). In 2015, the total expenditure of halal food and beverages was 1.17 trillion U.S. dollars, representing about 17% of the total global expenditure of 7 trillion US dollars according to the report of Global Islamic Economy. Halal market growth of this year was 3.4% from the year 2014, the value was slightly higher than the global trade growth of 3.3% (GIE, 2016). Global Islamic Economy also estimated that expenditure on the global food and beverage market projected to increase 1.9 trillion US dollars over 2021 (GIE, 2016). They also reported that China was the highest expenditure country on halal food accounted for US$ 854 billion, the second largest was United States with the figure of US$770 billion, followed by Japan with the value of US$380 billion and India with US$341 billion in 2015. On the other hand, expenditure on only halal food products was about 795 billion US dollars in 2014, but this expected to rise by 2.537 trillion over 2019 (Newshalal, 2016).

The US halal food trade is also rapidly growing, it was reported that halal foods were sold in about 200 shops in 1998 but the number rose to 7,600 at present (Green & Giammona, 2016). In 2015, the number of total Muslim was 3.3 million in the US, but the population projected to increase about 8.1 million over 2050 (Green & Giammona, 2016). Islamic Food and Nutrition Council of America (Halal certification authority) reported that halal sales from supermarkets and restaurants are projected to $US 20 billion

in 2016, increase to one-third from 2010 (Green & Giammona, 2016). US halal food sales have risen approximately 70% from 1995 (Nikfarjam, 2016).

Furthermore, according to Global Agricultural Information Network (GAIN) report in 2013 the estimated Muslim population in EU was 20 million and the expenditure on halal products was 30 billion, with the growth rate of about 15% and EU halal business expected to grow by about 100 million US dollar in the next year (Journo & Salmon, 2013). France is considered as a highest Muslim population county in Europe and accounted for approximately 4.7 million and its expenditure on halal products is about 8 billion US dollar. Moreover, the annual consumption of halal meat in French accounts for about 400,000 tons, representing 10-15% of the total meat of the country (Journo & Salmon, 2013). UK is another fast growing halal food market due to increasing Muslim population. According to the national census, the number of UK Muslim population increased from 1.55 million in 2001 to 2.71 million in 2011 (Evans, 2015). The estimated total expenditure of UK Muslims on food and beverages was 6.3 billion US dollars in 2014 and this is projected to increase at 5% per year over 2020 (Evans, 2015). In the UK more than 3,000 places are involved to supply the halal foods, for example, Subway offers halal foods at about 202 outlets in the UK, KFC has about 100 outlets for sale halal menu and Nandos also has 66 halal outlets (Evans, 2015; HexaResearch, 2016). Halal certified food products market also rapidly expanded in the Middle East. The United Arab Emirates established a Global center for Halal food accreditation to open in Dubai to ensure the standard of Islamic Sharia and certification of all the halal goods opening the UAE market and yearly imports of halal food will reach around 8.4 US dollars over the decade (Anonym, 2016a; KhaleejTimes, 2014). Total population is Muslim in Saudi Arabia and annual halal food trade is estimated at 5 billion US dollars (Jeddah, 2005).

Department of Islamic Development of Malaysia (JAKIM) has been playing an important role to implement the halal certification as well as monitor the halal food products and enforces the halal laws and regulations among the manufacturers, importers, distributors and restaurants since 1997. Malaysia has been well known globally as the international halal hub because the government established the Halal Development Corporation (HDC) for the control and support of halal the products in 2008 (GVR, 2017; Nikfarjam, 2016). Moreover, Malaysian government also developed Standards Malaysia (MS 1500:2004) to precisely describe particular guidelines for the preparation, production, storage and handling of halal food in 2004 and then revised in 2009 (MS 1500:2009) (Samori, Ishak, & Kassan, 2014). In 2015, the total halal export of Malaysia was 19.5 billion Malaysian Ringgit in the first half of the year with the growth of 3.6% from the first six months of 2014, reported by Malaysia External Trade Development Corporation Halal Unit (Rasid, 2016a).

Although Muslim, Christian and Jews are not strongly recommended vegetarianism from the religious aspect, but the religion of ancient India such as Hinduism, Buddhism and Jainism prefers the vegetarian lifestyle due to their religious obligation as well as their worshipful, respectful and compassionate to all other animal life (Davidson, 2003; Dudek, 2013). The impression on vegetarianism was strongest in Hindu religious people due to originated revering of the cow is a sacred animal. The vegetarianism in the Buddhism has arisen since the order of the Buddha to his followers for not killing the lives (Davidson, 2003). On the other hand, Jains adhere vegetarian diet because they belief that numerous nigodas are present in meat as well as in wine and honey and if they intake these items means involve in violent death which impede their rectification of the soul as they are comoletly ascetic (Davidson, 2003). It was estimated that the consumer of plant-based food about 4 billion worldwide (Pimentel & Pimentel, 2003).

2.2.4 Zoonosis

Zoonosis is the infection or disease that is naturally transmissible from animals to humans. According to Department of Agriculture and Fisheries (2016) of the Queensland Government over 200 zoonosis have been recognized these are caused by pathogenic agents such as viruses, bacteria, fungi, parasites and prions. Among these 13 zoonosis are more fatal because about 2.2 million people are died due to the infection of these pathogens (Bryner, 2021). Nowaday, endemic zoonoses cause an extra pressure of numerous diseases, specifically over the tropical zones and that also affect the livelihoods and food supply chain due to loss of livestock production (Halliday et al., 2015). Despite their detrimental affect, till date endemic zoonoses do not have proper recognition as well as understanding (Halliday et al., 2015). United States Department of Agriculture stated that about 60% of human pathogenic diseases are zoonosis and about 75% of infectious diseases are caused by animal origin (USDA, 2015). The livestock animals responsible for the maximum human zoonotic infections include cows, buffalos, pigs, goats, chicken, camels and sheep (Bryner, 2021). McDaniel et al., 2014 reported that until now about 45 pathogens responsible for bovine zoonosis have been identified and that are uniformly dispersed over the world. Among these the height percentages group is bacterial pathogens accounted for 42%, followed by 29% of parasitic organisms, 22% of viruses, 55 of fungi and 2% of prions (Figure 2.9).

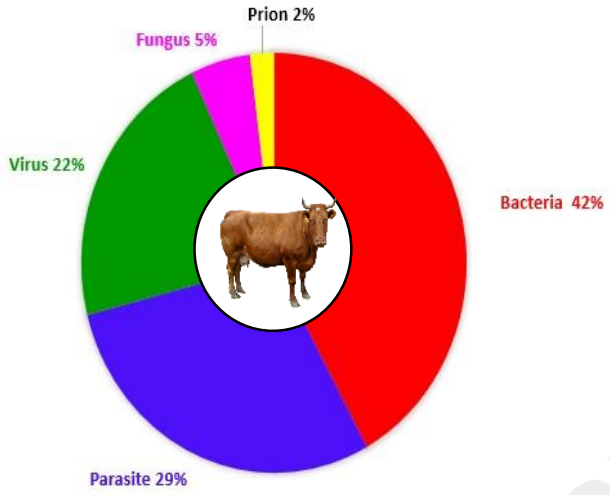

Figure 2.9: Cattle zoonotic bacterial pathogens (Adapted from McDaniel et al., 2014 with permission).

Among the zoonosis "mad cow disease" or bovine spongiform encephalopathy (BSE) is a fatal neurodegenerative disease, the origin of which is scrapie (spongiform encephalopathy) of goats and sheep that has been identified in Europe in the 18th century (Brown, Will, Bradley, Asher, & Detwiler, 2001). The BSE was first recognized in the UK in 1986 where it produced a serious outbreak, resulting about 170,000 cattle were infected, 4.4 million cattle were killed and a total of 164 people were died (Cleeland, 2009). As a result the national beef sale was fallen by 40% and international market sale was also decreased drastically as well as the significantly fell the cost of beef in UK (Buchanan, 2015). In 2003, consumption and export of beef were dramatically fallen in Belgium due to BSE (Goffaux et al., 2005). In 2003, BSE was also found in Canadian cattle, resulting the drastically fallen the beef export market and the Canadian beef manufacturers lost of about 5.3 billion dollars by 2004 (Sylvain Charlebois & Haratifar, 2015). There is no doubt about the human's susceptibility to the foot-and-mouth disease (FMD) virus because until now the FMD virus has been recognized in over 40 people cases. The symptoms in man are most likely that occurred in affected animals such as

developing vesicle on the feet and hands, particularly on the fingers, sometime it also appears on the palate and tongue of the mouth associated with fever (Bauer, 1997). The main host of the FMD virus is livestock animals, including cattle, water buffaloes, pigs, goats, yaks and sheep (Aftosa, 2014). Caron et al., 2016 (Caron, Cornelis, Foggin, Hofmeyr, & de Garine-Wichatitsky, 2016) shown that buffalo is another spreading factor of bovine tuberculosis and other zoonotic diseases. Buffalo is also a carrier of brucellosis pathogen, which is a serious disease causes infertility and affect both human and animals (Kats-korner, 2017; QLD.GOV, 2016). To stop the spread of brucellosis more than 3,000 buffaloes were killed in 1984 and about 1,000 buffaloes were killed between 1996 and 1997 in the Greater Yellowstone Area (Kats-korner, 2017). In addition, Buffalopox is another significant zoonotic disease of domestic buffaloes which caused reduced productivity as well as increased morbidity (Singh et al., 2006) and infect both cattle and human. In 2003, an epidemic of buffalopox occurred in Aurangabad, India associated with simultaneously infected to domestic buffaloes, cows and human (Gurav et al., 2011). Approximately 400 buffaloes were owned and total morbidity reached 45% of the affected buffaloes (Singh et al., 2006). Like cattle and buffaloes, pigs can also transmit several zoonosis to human being including swine influenza, Q fever, leptospirosis, Cryptosporidiosis, brucellosis, Rabies, Ringworm, Anthrax and Campylobacteriosis (KingCounty, 2016; Morrow & Langley, 2017). Huang et al., (2002) discovered swine Hepatitis E virus (HEV) in pigs of the United States and found that it has genetically closely similarity to the HEV of human. As a result, hepatitis E is now conceivable to be zoonosis. HEV is an outbreak in some countries, including the United States and made an important issues regarding public health (Huang et al., 2002). Some common zoonotic diseases associated with their carrier and route of transmissions is presented in Table 2.2.

Table 2.2: List of zoonosis (GOV.UK, 2013)

Disease	Causative agent	Host/ affected animals	Normal transmission mode to humans
Anthrax	*Bacillus anthracis*	Cattle, pigs, buffalo, sheep, horses, goats, dogs,	Direct contact, ingestion
Animal influenza	*Influenza viruses A, B and C*	Pigs, ducks, chickens, whales, horses, seals, and cats	May be reverse zoonosis
Bovine spongiform encephalopathy (BSE)	Prion protein	Cattle	Meat
Bovine tuberculosis	*Bacterium Mycobacterium bovis*	Cattle	Milk
Brucellosis	*Brucella melitensis* *Brucella suis* *Brucella abortus* *Brucella canis*	Cattle, buffalos, pigs, goats, sheep and dogs	Goats, sheep, pigs
Buffalo pox	Buffalopox virus	Buffalo and cattle	Direct contact
Campylobacteriosis	*Campylobacter jejuni*	Pigs and cattle	Direct contact and meat
Cysticercosis	*Taenia solium*	Cattle, buffalo, pigs	Meat
Cryptosporidiosis	*Cryptosporidium parvum*	Cattle, buffalos, sheep, pigs	Water, direct contact
Erysipeloid	*Erysipelothrix rhusiopathiae*	Pigs, fish, environment	Direct contact
Salmonella	*Salmonella enterica*	Cattle, buffalos, pigs, sheep, poultry	Foodborne
Haemorrhagic colitis	*Escherichia coli*	Ruminants	Direct contact and Foodborne

Table 2.2: continued

Disease	Causative agent	Host/ affected animals	Normal transmission mode to humans
Leptospirosis	*Leptospira* spp.	Cattle, buffalo, pig, dogs, horses, sheep and cats	Infected urine, water
Listeriosis	*Listeria monocytogenes*	Cattle, buffalos, pigs, sheep	Meat and dairy products
Q fever	*Coxiella burnetii*	Cattle, buffalo, pigs, sheep, goats, cats	Aerosol, direct contact, milk and fomites
Foot-and-mouth disease (FMD)	*FMD virus*	Cattle, buffaloes, pigs, sheep and goats	Direct contact and meat
Rabies	Rabies Virus	Cattle, dogs, foxes, bats, cats	Saliva (via bites or open wounds), direct contact with CNS tissue.
Rift Valley fever	*RVF virus*	Cattle, goats, sheep	Direct contact, mosquito bite
Streptococcal sepsis	*streptococcus*	Pigs, horses, cattle	Meat, direct contact
Swine influenza	Swine influenza virus	Pigs	Direct contact
Toxoplasmosis	*Toxoplasma gondii*	cattle, buffalo, sheep, goats and cats	Ingestion of fecal Oocysts, meat
Trichinellosis	*Trichinella parasite*	pigs, wild boar	Pork products
Zoonotic diphtheria	*Corynebacterium diphtheria*	cattle, farm animals, dogs	direct contact, milk

2.3 Current Species Detection Technique

Researchers have paid more attention to the development of ideal and precise technique for the detection of several animal species due to ever-increasing meat and meat products fraudulent issues worldwide (Ali et al., 2014c). Although morphological test is used for the identification of some food like honey, but it is not appropriate for the detection of meat species particularly in processed meat products (Cammà, Di Domenico, & Monaco, 2012). Moreover, microscopic technique also unsuitable for the meat product identification because it is unable to determine the accurate animal species in food staff (Ali et al., 2012d). However, numerous analytical approaches have been documented to detect the species origin in meat and meat products based on lipid, protein and DNA biomarkers. However, the lipid and protein based methods are often unsuitable because they are laborious, target-biomarker are often modified and thus cannot distinguish closely related species in highly processed food such as heated or chemically treated products, and are of less sensitive than DNA-based approaches (Ali et al., 2012a; Lago et al., 2011). Moreover, these methods are unable to differentiate closely related species, such as cow and buffalo. In contrast, the DNA-based techniques, especially the short-length DNA biomarkers are thermodynamically more stable, more sensitive and more reliable over the longer ones even under extreme states such as degraded or naturally decomposed samples (Ali et al., 2015b; Rashid et al., 2015a). The field of use and limitation of these methods are briefly presented here.

2.3.1 Lipid Based Assay

Lipid based techniques for analysis of meat species involves in the analysis of fatty acids positional distribution in triacylglycerol (TAG) and 2-monoacylglycerol (2-MAG) as all species stored n-6 polyenoic and monoenoic fatty acids in TAGs with unsaturation (except pigs) at the sn-2 position and larger chain length (SzabO, FEBel, SugAR, & RomvARi, 2007). SzabO et al., (2007) reported that rabbit and ruminants contain high amount of odd-chain-length fatty acids in their native TAGs which are the detectable markers of these two species. On the other hand, pigs can be detected by the analysis of 2-MAGs because they contain lower unsaturation in 2-MAGs. However, measurement of the fatty acid positional distribution provides information for the identification of the species but the content and varieties of the TAGs and 2-MAGs usually modified due to the processing and cooking treatments. Thus, these methods have very limited used for the identification of species in food and foodstuff due to its less reliability.

The Fourier transform infrared spectroscopy (FTIR) together with partial least square (PLS) or principal component analysis (PCA) is an important tool for the authentication of food species based on lipid (Rohman, Sismindari, Erwanto, & Che Man, 2011). Infrared absorption spectrum of the samples were measured in the FTIR assays and this method is also able to collect high spectral resolution data (Griffiths & De Haseth, 2007). Analysis of fatty acids is important for the differentiation of fats from animal and plant sources. Therefore, analysis of fatty acids plays an important role in identification of adulteration or replacement of vegetable oils with lower priced lard in Kosher, halal and vegan food products.

2.3.2 Protein Based Assay

An overview of protein based assays for the detection of species in meat and meat products are described below:

2.3.2.1 Histidine Dipeptides Based Assay

Animal tissues, namely muscle, heart, kidney and liver naturally contain some dipeptides associated with histidine such as balenine (β-alanyl-L-3-methylhistidine, ophidine), anserine (β-alanyl-L-1-methylhistidine) and carnosine (β-alanyl-L-histidine). These dipeptides play an important physiological role in the tissue, such as antioxidant, buffering, vasodilatory activity, neurotransmitter action and enzyme modulator (Aristoy, Soler, & Toldrá, 2004; Carnegie, Hee, & Bell, 1982). Histidine dipeptides are present only in animal tissues, but not in plant sources and these dipeptides are also animal specific (Aristoy et al., 2004). Thus, the species origin can be detected in the processed meat products by determining the ratio of these dipeptides particularly the ratio of carnosine and anserine or vice versa, because histidine dipeptides remain unaffected by heat treatment (Aristoy & Toldrá, 2004; Tinbergen & Slump, 1976). For example, Aristoy and Toldra (2004) shown that the height ratio of carnosine and anserine was in pork with 17.88±3.74, followed by beef with 8.08±1.91, lamb with 0.95±0.26 and poultry with 0.20±0.08. On the other hand, Tinbergen and Slump (1976) determined the ratio of anserine and carnosine for beef, pork and chicken and the results were found to be between 0.06-0.2, 0.02-0.1 and 2.2-5.5, respectively. Therefore, by measuring of these dipeptides can easily identify the existence of animal protein in feedstuff, as plant sources do not contain these dipeptides. This method was particularly developed for the detection of animal proteins in the animal feeds. Because bone meat meal, meat meal, fish meal etc. are the main source of calcium, amino acids and phosphorus, which play a role in the rapid growth of farmed animals (Aristoy & Toldrá, 2004). But due to the Prevalence of mad cow disease (BSE), the use of animal proteins was forbidden in the

feed of ruminants in worldwide (Aristoy et al., 2004; Aristoy & Toldrá, 2004). Although, this technique can identify the origin of mammalian but is unable to determine the specific animal species, especially in complex matrices of various species (Aristoy & Toldrá, 2004), reflecting the requirement of more specific and precise method for this analysis.

2.3.2.2 Analysis of Muscle Protein

Muscle protein can be originated by using isoelectric focusing (IEF) electrophoresis. Muscle proteins present in the sarcomeres or sarcoplasm are the target for the authentication of the species origin. The cytoplasmic part of the muscle cell (myocyte) is sarcoplasm and the structural unit of the muscle fibers (myofibers) is sarcomere (Hulland, 1993). Parvalbumins are present in high concentration in the fish muscle sarcoplasm, which are small, calcium-binding, acidic and heat-stable proteins. As these proteins are species specific and isoelectric P^H range is 3.8 to 5.3 in native state, the IEF profile of these proteins have been effectively introduced to discriminate the fish species (Addis et al., 2010; Berrini, Tepedino, Borromeo, & Secchi, 2006). Berrini et al. (2006) revealed that IEF profile is able to differentiate the inter-species polymorphic species but is not suitable for intra-species polymorphic species. Thus, two-dimensional electrophoresis (2 -DE) can overcome this problem. 2-DE map of myosin light chain (MLC), a sarcomeric protein, can clearly distinguish the fish species as well as able to provide information of the preserve condition and freshness of the specimens (Martinez & Jakobsen Friis, 2004). Moreover, 2-DE method couple with proteomic assay, namely mass spectroscopy and in-gel digestion, are more suitable tool for discriminating the species specific MLC in admixed and processed samples of different tissues and muscles of various species (Martinez & Jakobsen Friis, 2004; Pischetsrieder & Baeuerlein, 2009). Giometti et al., (1979) proposed that high-resolution two-dimensional electrophoretic technique can feasibly be applied for the analysis of biopsy samples of human muscle by resolving the

major muscle proteins and enzymes. They successfully identified the ten enzyme components and actin, myosin, troponin and tropomyosin from the two-dimensional profile using rabbit muscle as a model. By comparing the human and rabbit muscle patterns found enormous similarities, but not confirm identifiable and additional modification is required for final results (Giometti et al., 1979). Thus, electrophoretic and proteomics techniques are expensive, required skilled technicians, laborious and also not suitable for the investigation of admixed samples of different species (Addis et al., 2010; Martinez & Friis, 2004; Pischetsrieder & Baeuerlein, 2009).

2.3.2.3 Analysis of Species-specific Osteocalcin

Osteocalcin (γ-carboxyglutamic acid-containing protein) is noncollagenous protein found in bone and dentin of most animals and play role in the formation of bone. According to EU Regulation EC No 999/2001 (EC, 2001) feed containing meat and bone meal (MBM) is restricted for farmed animals. Furthermore, addition of animal proteins in the feedstuff of same species is also prohibited under the Regulation EC No 1774/2002 (EC, 2002). The permitted MBM source is only fish meal in the feed of fowl, pig and calves (EC, 2001). Thus, analysis of feedstuff to detect the contaminated animal MBM is mandatory by the EU Regulation EC No 999/2001 and EC No 1774/2002 (EC, 2001; EC, 2002). The classical optical microscopic technique is the accepted official method for the identification of MBM in the feedstuff (EC, 2009). This method is reliable for the detection of animal origin, which are stable under processing treatment (133^0 C and at 300 kPa for 20 min) required for MBM manufacturing, such as bone fragments, scales, gills teeth or hair (Kreuz et al., 2012). But microscopic method cannot apply in the quantitative approaches and to overcome this limitation spectroscopic (near infrared spectroscopy—NIRS) method was introduced (Abbas et al., 2010). To increase the performance of the spectroscopic method for analyzing the animal proteins in feedstuff, near infrared microscopic (NIRM) method has been developed. The NIRM is more useful

because it possesses both spectroscopic and microscopic functions in one instrument. The recent modified form of NIRM is NIR hyperspectral imaging, which allows both spectral and spatial characterizing information of the specimen simultaneously. The sensitivity of the NIRM methods is up to 0.5% level of adulteration in feed specimen (Abbas et al., 2010). In addition, Fourier transform near infrared spectrometer (FT-NIR) couple with auto image microscope also have significant role for the differentiation of species contaminated in feedstuff. Haba et al., (2007) developed FT-NIR microscopic method for the discrimination of land-animal and fish particles in feed samples.

The protein, osteocalcin (OC) is a not suitable target molecule for the differentiation of species due to its conserved nature as well as very low variability in the sequences. But there is enough variation at the genus level of OC such as it contains amino acid sequence variation between the species which help to distinguish the different species (Balizs et al., 2011). Consequently, Balizs et al., (2011) developed a suitable method for detecting species-specific OC on the basis of mass differences due to the variation in amino acid sequences, by using the matrix-assisted laser desorption ionization/time-of-flight (MALDI/TOF) and high-resolution hybrid mass spectrometry (HR-Q/TOF MS). This method was successfully applied for the differentiation of bovine and porcine materials in MBM samples (Balizs et al., 2011). In addition, Kreuz et al., (2012) developed sandwich ELISA technique to identify the MBM in feed, on the basis of raising antibody against the bovine osteocalcin. The developed method is stable under the heat treated samples (145^0 C) and is very sensitive (1 ng for pure state and 0.1% for adulterated sample) and they also proposed that it may apply for the discrimination of bovine and horse species (Kreuz et al., 2012). However, these methods are highly expensive, required skilled operator to operate and unable to differentiate specific-species properly, particularly in the mixed matrices.

2.3.2.4 Detection of Species Specific Proteins by ELISA

Although above described protein-based methods are suitable for the identification of feed and food ingredients but these are not applicable for the routine analysis of commercial feed and food products because they are comparatively expensive, laborious, complex to handle and time consuming (Asensio, González, García, & Martín, 2008). On the other hand, the immunological method, namely Enzyme-Linked Immuno Sorbent Assay (ELISA) has been more suitable and widely used tools for the authentication of food products due to its low cost, high specificity, sensitivity and simplicity (Asensio et al., 2008; Carrera et al., 2014).

The principle of ELISA is that either antibody (Ab) or antigen (Ag) is fixed to a surface followed by the measurement of antigen-antibody interactions by the assistance of the labeled enzyme (E) which converts specific substrate into a colored product. The measurement of the produced color is an indicator for the identification and quantification of the sample (Pokhrel, 2015). The most commonly used ELISA techniques for the authentication of feed and food products include indirect and sandwich ELISA.

(a) ***Indirect ELISA***

This method comprised of two steps, here additional one set of antibodies is used compared to direct ELISA. The initial step involves antigen specific antibody (primary antibody) bind with the fixed antigen and the second step involves binding of enzyme labeled secondary antibody, which is primary antibody specific, to the primary antibody. The antibody conjugated enzyme react with the suitable substrate produces color product. Another form of indirect ELISA is Sandwich ELISA (Asensio et al., 2008).

$$\text{Ag} + \text{Ab} + \text{Ab-E} \xrightarrow{\text{Substrate}} \text{Color product}$$

(b) ***Sandwich ELISA***

Here, antigen is bounded by two different antibodies, one is capture antibody which remain fixed with solid surface and another is detection antibody labeled with enzyme. The analytical antigen must contain minimum two antigenic epitope able to bind with antibody as two antibodies participate in Sandwich ELISA. Among the immunosorbent assays Sandwich ELISA is most useful tool because of its high antigen detecting efficiency and sensitivity than those where antigen is fixed to the solid surface. In addition, no sample purification is needed before analysis with Sandwich ELISA (Asensio et al., 2008).

$$\text{Ab} + \text{Ag} + \text{Ab-E} \xrightarrow{\text{Substrat}} \text{Color product}$$

ELISA assay can be used in both qualitative and quantitative analysis. The development of antibodies against the target antigens is mandatory for the ELISA analysis. Two types of antibodies are used in the ELISA technique to authenticate the food ingredients, namely monoclonal (Chen, Hsieh, & Bridgman, 1998; Hsieh & Bridgman, 2004; Liu, Chen, Dorsey, & Hsieh, 2006) and polyclonal (Berger, Mageau, Schwab, & Johnston, 1987; Hsu, Pestka, & Smith, 1999; Smith et al., 1996) antibody. Polyclonal antibodies (PAbs) are more suitable for the analysis of denatured protein samples as they are able to recognize the antigens from a mixture of different epitopes and with little changes in the property of antigen, such as denaturation or polymerization. However, PAbs have some limitations including limited yield, variable affinity and extensive purification step is needed to overcome cross-reactivity for the detection of specific-species. On the contrary, MAbs are produced homogenously by using hybridoma techniques with high yield, specific biological activity and high specificity (Asensio et al., 2008).

Until now, various reports have been documented for the authentication of food using both MAbs and MAbs on the basis of structural and soluble proteins of the muscle cell. To detect the adulterated pork in beef mixture, Sandwich ELISA technique was introduced by raising PAbs against muscle soluble protein with the detection limit of 1% adulteration level (Martín, Azcona, Casas, Hernández, & Sanz, 1988). ELISA technique was also used for the authentication of food products, such as fermented sausage, cooked salami and frankfurter (Ayaz et al., 2006) and hamburger (Macedo-Silva et al., 2000).

ELISA method was introduced for the quantitative measurement of the raw pork in the admixture of raw beef with the quantification limit up to 1% (Martin, Chan, & Chiu, 1998). Chen and Hsieh (2000) reported quantitative ELISA technique for the quantification of pork in heat treated various meat products such as sausage bologna ham, salami spread franks and luncheon meat using MAbs which was raised against heat-stable muscle protein of pig. The limit of detection was found 0.5% (w/w) porcine material in various meat mixture and the accuracy of the developed method was confirmed by comparative study with commercial PAbs test kit. Similarly, Liu et al., (2006) developed MAbs based quantitative Sandwich ELISA assay for the evaluation of porcine material in thermal-treated (132^0 C for 2 h) meat samples with the lower detection limit of 0.05% of pork in adulterated mixture. Currently, the ELISA test kits of specific meat species are commercially available for the reliable analysis of raw, processed, cooked meat, meat product and feedstuff (Asensio et al., 2008).

More recently, Perestam et al. (2017) reported a comparative study between ELISA and DNA-based method (real-time PCR) based on specificity, sensitivity, time, cost and purpose of use. They found that both methods are suitable for detecting the species origin in raw meat and meat products but ELISA is not suitable for the identification of species in highly processed food particularly when a lower detection limit is requisite. Other

researchers also concluded regarding the lower sensitivity of the ELISA assay and also not suitable for the differentiation of species in mixed matrices particularly in closely related species (Martin et al., 1998; Martín et al., 1988). Moreover, immunoassays often interrupted due to cross-reactions occurrence between closely related species, since these techniques are based on the raised of antibodies against a specific protein (Di Pinto et al., 2005; Fajardo et al., 2010).

2.3.3 DNA-based Method

Recently, researchers have paid more attention to the DNA-based methods and these methods becoming more prominent and widely used for the verification, quantification and monitoring of adulterated species in meat and meat products because of its specificity, sensitivity, preciseness, robustness, rapidity and inexpensiveness (Darling & Blum, 2007). The DNA-based methods are considered as highly useful tools in practical fields due to the exceptional properties of DNA molecule such as codon degeneracy, superior heat stability, abundant presence in multiple copies in most cells along with intra-species conserved and inter-species polymorphic fingerprint etc. (Ali et al., 2014c; Mafra et al., 2007). Stability of biomarkers is a key factor for successful species detection particularly in processed meat products, as these products are prepared under extreme heat and processing treatment. Unlike protein biomarkers which readily denature under heat processing treatment, DNA biomarkers are highly stable under severe processing condition (Mane, Mendiratta, Tiwari, & Bhilegaokar, 2012). Furthermore, a small amount of sample is enough for the detection species in DNA-based methods because multiple copies of DNA are present per cell (Gupta et al., 2011; Mane, Mendiratta, & Tiwari, 2012). In addition, DNA also carry enormous information compared to proteins due to the genetic code degeneracy and the existence of large non-coding stretche (Pereira, Carneiro, & Amorim, 2008). Due to the above advantages, DNA-based methods have become more favorable tool for the detection of species in complex background of heavily

processed foods. However, among the DNA based assay, PCR has been gained increasing attention due to accuracy, higher sensitivity, reliable and rapid investigation scheme, where DNA is used as a detection target and a single DNA copy is amplified into multiple copies (Aida, Man, Wong, Raha, & Son, 2005; Ali et al., 2014c).

Design of specific biomarkers of the target species is a fundamental step of PCR assay development. According to the research requirement, both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) have been introduced in numerous studies for the design of biomarkers (Morin, Hedrick, Robertson, & Leduc, 2007). Researchers have gained particular attention to the mitochondrial DNA (mtDNA) over nuclear DNA (nDNA) especially, for the identification of meat products due to the following advantages:

(i) the absence of pseudogene or repetitive sequence, complicated intron which result in simpler in complexity than nDNA,

(ii) rapid evolution of mtDNA due to the higher base substitution rate than nDNA, allowing the existence of more diversity in sequences and facilitating the differentiation of phylogenetically closely related species (Fajardo et al., 2010; Zha et al., 2010),

(iii) sequence of mtDNA is more conservative because of its maternal inheritance and lack of recombination in all vertebrates (Rokas, Ladoukakis, & Zouros, 2003)

(iv) more stable because mtDNA is present in higher number per cell (800-1000) and surrounded by double membrane (Cooper, 2000; Girish et al., 2004).

Thus, mtDNA can survive under severe processing treatment, offering the target of biomarker design for the reliable detection of species in compromised samples and in the admixture of closely related species (Ali et al., 2011b; Karabasanavar et al., 2014; Mane et al., 2012).

2.3.3.1 Polymerase Chain Reaction (PCR) Based Assay

PCR is an in vitro process in which a specific target DNA fragment can be amplified from a single or small number of DNA to a large number of DNA under a simple enzymatic reaction (Garibyan & Avashia, 2013).

The major components of the PCR reaction include, primers, template DNA, DNA polymerase and nucleotides (Garibyan & Avashia, 2013). Only simple three-steps cycling reactions are required for PCR assay, such as

(i) Double stranded DNA denaturation

(ii) Primers annealing

(iii) Primer extension

When amplification target is RNA, a complementary DNA (cDNA) of that RNA must be generated with the help of reverse transcription prior to PCR is started (Schochetman, Ou, & Jones, 1988). The key function of the PCR reaction is the association of individual building blocks nucleotides (adenine, guanine, cytosine and thymine) together by the enzymatic reaction of DNA polymerase for the synthesis of PCR products. The primers are short single stranded DNA sequences and complementary to the DNA of target species either from 5'-end or 3'-end of the desired sequence. Annealing of the primers with the dissociated DNA stands facilitate the DNA polymerase to start the extension of new stands. Thus, after completion of each cycle, the copy of DNA is become double, allowing the synthesis of large number of DNA after 30 to 40 cycles. After mixing the PCR all PCR reagents in the PCR tube or 96-well plate is placed in the Thermal Cycler to run the three basic steps of repeated DNA amplification reaction (Figure 2.10) (Garibyan & Avashia, 2013; Schochetman et al., 1988). For the detection of PCR amplified products, DNA visualization is accomplished under an electrophoresis system of agarose gel or polyacrylamide by staining with ethidium bromide or other non-

carcinogenic DNA stain (eg. Florosafe DNA stain) and an appropriate DNA size marker under a gel image documentation system for only gel image (Lee, Costumbrado, Hsu, & Kim, 2012) or on automatic Capillary Electrophoresis System for both gel image and electroferogram (Dooley et al., 2005; Fajardo et al., 2010). Among the DNA-based studies, PCR assays have occupied the central place because they can amplify a specific fragment of DNA from a minute quantity such as single copy to any detectable quatities (Reid, O'donnell, & Downey, 2006). Because of this feature a large number of PCR methods have been developed for the authentication of different species such as fish and meat species. A brief description of the different PCR-based assays is illustrated below under different subheadings:

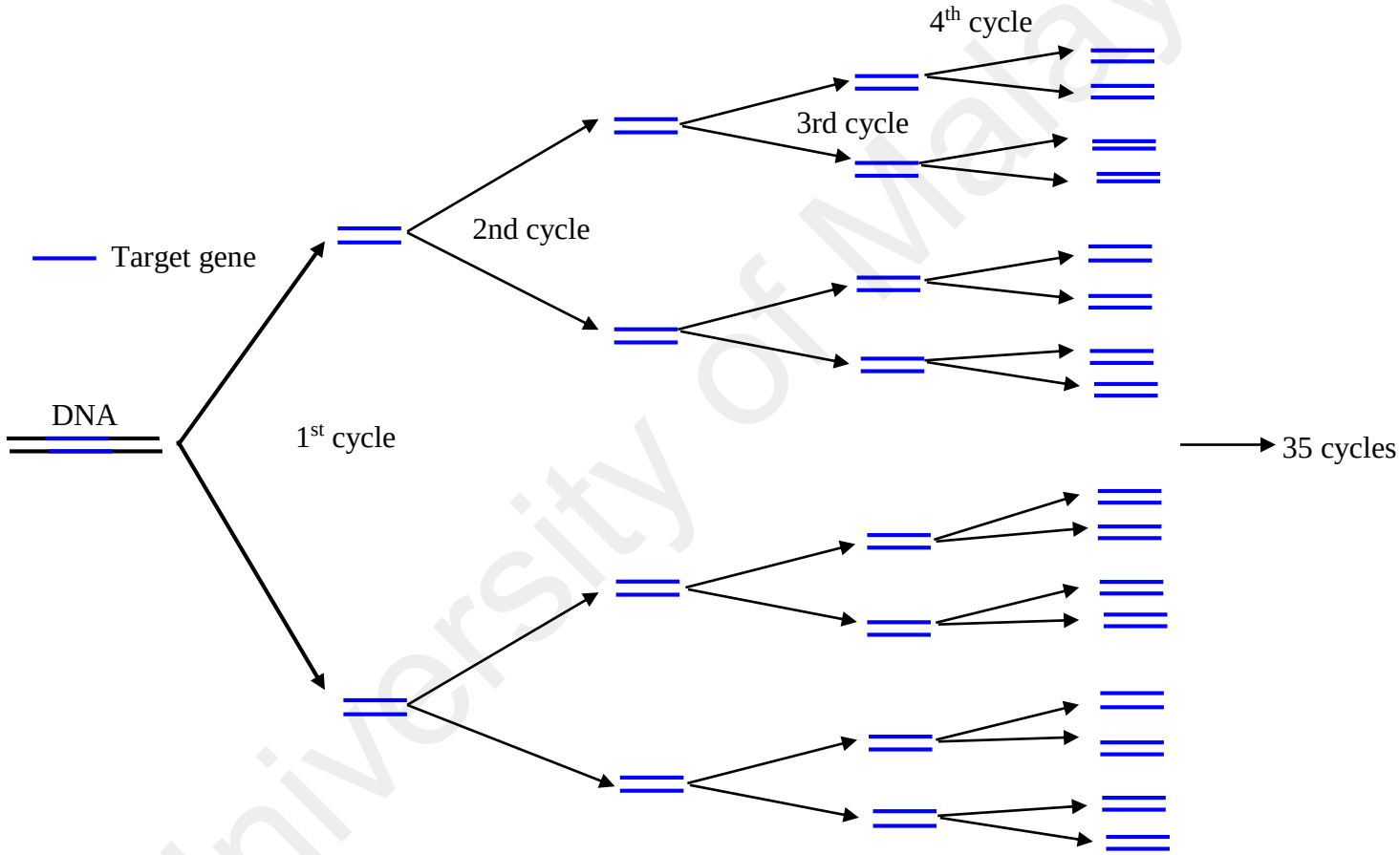

Figure 2.10: Amplification of target gene by PCR reaction (Adapted from Vierstraete, 1999)

(a) ***PCR Sequencing***

DNA barcoding was introduced in 2003 and it has been applied as a reliable, fast and inexpensive method that can identify species without necessitating taxonomic analyses (Luo et al., 2011; Vernooy et al., 2010). DNA barcoding often amplifies about 650 bp fragment of mitochondrial cytochrome oxidase I (COI) gene and assign species based on sequence variation to make reference sequences that can act as a molecular detection tag for each of the species profiled by PCR (Fajardo et al., 2010). Identification of species are usually accomplished by comparing the sequences of target species with DNA barcodes of known species through alignment searching, distance based tree construction, decision theory, the characteristic attribute organization system and the back propagation neutral network (Luo et al., 2011).

A Canadian national research network has developed the Barcode of Life Data Systems (BOLD) (http://www.boldsystems.org) which currently accommodates barcode records for over 850,000 samples, representation about 100,000 species (Vernooy et al., 2010). The invention of DNA barcoding system seems to be promising in various area like forensic analysis, biosecurity and food authentication as well as protection of wildlife (Ferri, Alu, Corradini, Licata, & Beduschi, 2009). Most of the studies regarding food speciation using DNA barcoding system have focused on fishery and seafood products (Fajardo et al., 2010). For example, Barbuto et al. (2010) applied DNA barcoding method for the detection of shark slices sold (palombo) using 550 bp barcode sequence from coxI gene. The developed technique was able to identify adulteration in 80% of the tested samples of commercial palombo in Italy. Another approach for the authentication of seafood was introduced by Wong et al. (2008); wherein they used 652 bp sequence from the COI gene and successfully identified that 25% of the specimens were potentially mislabeled. Recently, Hajibabaei et al. (2006) developed a short length barcode (~100 bp) for the identification of museum specimens, as higher length barcode like 650 bp

cannot recover with full length due to DNA degradation in highly decomposed samples. DNA barcode system was also developed for the detection of domestic animals. Ramadan, (2011) designed one set of universal primer targeting 422 bp mitochondrial 16S rRNA gene of buffalo. The developed system successfully identified buffalo as well as cattle, goat and sheep.

Although DNA barcoding system has gained wide spread support in the identification of species and biodiversity screening, it is not free from limitations. Firstly, DNA barcoding amplify long DNA fragment, like 650 bp segment of COI gene which often breakdown in heat and pressure treated foods and feeds samples (Ali et al., 2015c; Fajardo et al., 2008; Hird et al., 2006). Secondly, the system is applicable for only single species detection scheme and cannot be applied for the detection of multiple species in a single assay platform. Thirdly, this technique require two major steps: one is PCR amplification and second one is the post-PCR sequencing of the amplified products reflecting that make it quite expensive. Fourthly or finally, the assay cannot generate quantitative data (Ali, 2011a).

(b) ***Species Specific PCR***

Recently, researchers have paid more attention to the species specific PCR (SSP) targeting mitochondrial genes due to its simplicity, sensitivity, preciseness, cost-effectiveness and requirement of very lower amount of sample (Rashid et al., 2015b). In this method, target DNA fragment is amplified using one set of primers (forward and reversed) by an enzymatic reaction of DNA polymerase followed by separation on agarose or polyacrylamide gel with ethidium bromide or other non-carcinogenic staining dye to visualized (Ali, 2011a). Both simplex /singleplex (Barakat, El-Garhy, & Moustafa, 2014; B. G. Mane et al., 2012) and multiplex (Dalmasso et al., 2004; Hou et al., 2015) SSPCR assays have been documented.

i ***Singleplex PCR***

The method involves amplification or detection of single species in a reaction. Until now, enormous simplex PCR assays for the detection of various species with different target (amplicon) sizes have been documented due to its sensitivity, accuracy and robustness. For example, Mane et al. (2012) introduced beef specific PCR assay based on 513 bp amplicon sized from mitochondrial D-loop gene for the detection of raw, processed and autoclaved beef and beef products. Arslan et al. (2006) also reported beef specific PCR assay for the identification of various heat treated meat including boiling, pressure cooking, roasting and pan frying by amplifying 271 bp fragment of mitochondrial DNA. Various reports have also been documented for the authentication of buffalo species. Girish et al. (2013) developed a rapid detection method of buffalo species using mitochondrial D-loop gene for amplifying the 482 bp fragment. Another highly specific PCR assay was developed targeting the same gene for the identification of buffalo meat which amplified 534 bp PCR product (Karabasanavar et al., 2011). Kumer et al. (2011) reported buffalo mitochondrial D-loop specific PCR assay targeting 358 bp amplicon size. To authenticate the processed meat and meat products, a buffalo specific PCR assay was documented for the amplification of 537 bp amplicon from mitochondrial D-loop gene. The assay was sensitive up to 1% level of adulteration under autoclaved condition (Mane et al., 2012). Recently, Vaithiyanathan et al. (2016) developed beef and buffalo specific PCR methods with a common forward primer for both beef and buffalo and the species specific reverse primers from the mitochondrial D-loop region. The developed systems successfully amplified 126 bp and 226 bp PCR products for beef and buffalo species, respectively with a detection level of 0.47 ng for beef and 0.23 ng for buffalo DNA in simplex PCR assays. Numerous simplex PCR assays also introduced for the verification of porcine material in food chain. To developed pork specific PCR system, different types of mitochondrial genes have been targeted with different amplicon

sizes including cytb (Aida et al., 2005; Ali et al., 2011b), D-loop (Man, Mustafa, Mokhtar, Nordin, & Sazili, 2012; Haunshi et al., 2009; Karabasanavar et al., 2014) and 12S rRNA (Man et al., 2007). Other species also detected by using simplex PCR assay such as goat (Kumar, Singh, Singh, & Karabasanavar et al., 2011a; Rodríguez et al., 2004), sheep (Rodríguez et al., 2004), cat (Ali et al., 2015b), dog (Rahman et al., 2014), monkey (Ali et al., 2016) and turtle (Ali et al., 2015c).

ii ***Multiplex PCR***

The multiplex PCR is a highly useful and remarkable technologies, where multiple target DNA fragments are amplified simultaneously in a single assay mixture, reducing both time and cost (Hou et al., 2015). Both conventional (end-point) and real-time PCR assay have been introduced for the authentication of meat and meat products. Nowadays, these techniques have got great promise since they offer abundant advantages. Matsunaga et al. (1999) were the first to introduce multiplex PCR technique for the detection of five meat species such as pig, cattle, goat, horse and sheep. They used a common forward primer from the mitochondrial cytb gene and reversed primer from species specific DNA sequences. Rea et al. (2001) developed a duplex platform for the detection of bovine and water buffalo milk and mozzarella cheese based on 113 bp and 152 bp fragments from cytb gene of bovine and water buffalo respectively. The sensitivity of the method was found to be 1 pg for raw and 1% level for adulteration. Gupta et al. (2012) optimized the same primer pairs which were developed by Rea et al. (2001) for the simultaneously detection of beef and buffalo meat with the similar sensitivity (1 pg). Duplex PCR was also introduced for the authentication of cattle and buffalo fat targeting mitochondrial D-loop gene of both species. 126 bp and 226 bp PCR products were successfully amplified for cattle and buffalo respectively and the limit of detection was 0.12 ng for buffalo 0.47 ng for cattle (Vaithiyanathan & Kulkarni, 2016). Bai et al. (2009) developed a multiplex

PCR assay for the detection of cattle, pig, chicken and horse meats by amplifying 292, 412, 239 and 451 bp fragment, respectively. The sensitivity of the assay was found to be 0.1 ng. Multiplex PCR was also developed for the analysis of feedstuff to detect the species commonly used in rendering plants namely, ruminant, pork, poultry and fish. To carry out the authentication 104, 290, 224 and 183 bp PCR products of the mitochondrial genes (16s rRNA for ruminant and 12S rRNA for others) were amplified with detection limit of 0.002% for ruminants, pork and poultry and 0.004% for fish (Dalmasso et al., 2004). Mitochondrial cytb gene was targeted for the amplification of 398 and 439 bp sequences to identify pig and horse respectively in a single assay platform (Di Pinto et al., 2005). He et al. (2015) optimized multiplex PCR technique to detect four different species including pork, beef, duck and mutton. The identification was carried out by using 212 (pork), 116 (beef), 322 (duck) and 177 (mutton) bp fragments from cytb, cytb, ND2 and 16S rRNA, respectively. Recently, Ali et al. (2015d) developed a multiplex PCR method for the simultaneous identification of five species forbidden in Halal (Islamic) foods, such as pig, dog, monkey, cat and rat. The targeted genes were mitochondrial cytb for cat, ATPase 6 for rat and dog and ND5 for monkey and pig, for the amplification of 172, 108, 163, 129 and 141 bp DNA fragments respectively. Multiplex PCR also extended for the verification of genetically modified organisms (GMOs) in food and feed (Germini et al., 2004).

Thus, multiplex PCR assay is highly promising and useful technique discriminatory power of identifying several species under complex matrices. Thus it can save both labor and time. On the other hand, simplex PCR assay needs several different assays since each set of species specific biomarkers are used separately (Zha, Xing, & Yang, 2011). However, all of these assays are based on single gene targeted and most of them are long DNA targeted which are not suitable for the analysis of highly degraded samples due to the breakdown of the target amplicon.

iii ***PCR- Randomly Amplified Polymorphic DNA (PCR-RAPD)***

Randomly Amplified Polymorphic DNAs (RAPD) are the fragments of DNA that are amplified by PCR assay with the help of synthetic short oligonucleotide primers complementary to random sequence. Therefore, PCR-RAPD method involves in the simultaneously amplification of many distinct DNA fragments due to the randomly binding of the single arbitrary short primer (generally 10 bp) at the many different location on the genomic DNA followed by carry out the gel electrophoresis for the separation and visualization of the amplified products depending on their sizes (Fajardo et al., 2010; Hadrys, Balick, & Schierwater, 1992). Samples identification are accomplished by comparison the DNA bands profile according to the expectation depending on experimental conditions, primer and DNA used as the produced band pattern from amplified products are characteristics of the template DNA (Fajardo et al., 2010; Kumar & Gurusubramanian, 2011b).

Arslan et al., (2005) used PCR-RAPD technique for the identification of various animal species in raw and processed meat products. The method successfully identified the cow, pig, sheep, goat, wild swine, camel, dog, cat, donkey and rabbit or bear species using a short (10 bp) primer. The method is also applicable for the detection of species origin in the 1:1 mix of raw minced meat from beef-sheep, horse-beef or sheep-pork. This method was also applied for the detection of ten meat species namely beef, buffalo, pig, wild boar, horse, cat, dog, venison, kangaroo and rabbit by producing fingerprint patterns using 10 bp containing 29 primers. Although, some primers of this method can generated district fingerprints for the differentiation of the species but other cannot distinguish the species origin (Koh, Lim, Chua, Chew, & Phang, 1998). Martinez et al., (1998) applied this technique for the authentication of beef, buffalo, pork, goat, elk, mule, ostrich, donkey, reindeer, kangaroo, horse, and lamb species in the various meat products such as

frozen red meat, sliced, salmoni and Lammerull. Another approaches of PCR-RAPD for the identification of four meat species including cattle, buffalo, sheep and goat (Calvo, Zaragoza, & Osta, 2001a). Single arbitrary primers containing PCR-RAPD technique have some advantages including simple, rapid, eliminating more complex analytical steps and no need previous knowledge of the target DNA sequence (Fajardo et al., 2010). However, the main limitation of this method is reproducibility, in practice it is very difficult to produce reproducible amplified DNA band pattern (Arif et al., 2010; Koh et al., 1998). Furthermore, this technique is not applicable for the analysis of extremely processed meat and meat products, as highly purified DNA is mandatory for the reproducible RAPD patterns. In addition, PCR-RAPD method is not suitable for the identification of species in mixed samples containing more than one species (Fajardo et al., 2010).

iv ***PCR-restriction fragment length polymorphism (PCR-RFLP)***

PCR restriction fragment length polymorphism (PCR-RFLP) is one of the most important molecular techniques accomplished by numerous researchers. The PCR-RFLP assays are especially interesting because they offer the opportunity to authenticate a product by restrictive digestion of the amplified PCR products using one or more restriction enzymes (REs) (Rashid et al., 2015b). Using the sequence variation that exists within a defined region of DNA, the differentiation of even closely related species is possible using a PCR-RFLP assay (Hsieh & Hwang, 2004). However, the PCR-RFLP technique is very simple and inexpensive and easily applicable in the routine analysis (Farag, Alagawany, El-Hack, Tiwari, & Dhama, 2015).

Species-specific PCR assay is often conclusive but it has yet to be considered a definitive analytical method because of certain “hard-to-control” features of the amplification process (Focke, Haase, & Fischer, 2010; Yang, Kim, Byun, & Park, 2005).

For example, it sometimes produces artifacts due to contamination by alien DNA at a minute scale (Doosti, Dehkordi, & Rahimi, 2014; Yang et al., 2005), but these ambiguities or doubts could be eliminated by the verification of the amplified product through at least one of three different methods, namely, PCR-RFLP assay, probe hybridization, and target product sequencing (Maede, 2006). Probe hybridization is an attractive technique because it can detect multiple species in a single experimental run through the use of multiple labeled probes, (do Nascimento, de Albuquerque, Monesi, & Candido-Silva, 2010) but this procedure requires purified DNA and is also laborious, expensive, and time-consuming (Rashid et al., 2015b). In contrast, DNA sequencing is a more efficient and reliable tool, but it requires an expensive laboratory setup and is often not suitable for the analysis of processed food under complex matrices (Girish et al., 2004; Mafra et al., 2007) because of the coextraction of the food ingredients that often bring errors into the final results (Times, 2015). On the contrary, the PCR-RFLP assay can overcome all of these limitations and has been widely used to authenticate the original PCR product amplified from a particular gene fragment (Park, Shin, Shin, Chung, & Chung, 2007; Sharma, Thind, Girish, & Sharma, 2008). It comprises the generations of a specific fragment profile through restriction digestion with one or two endonucleases. A carefully selected restriction endonuclease cleaves the PCR product at specific recognition sites, producing a set of DNA fragments of different lengths that could be separated and visualized by gel electrophoresis (Ballin, Vogensen, & Karlsson, 2009); thus, it distinguishes the artificial PCR product from the original through the analysis of the restriction fingerprints (Doosti et al., 2014; Times, 2015).

Such assays have been successfully applied to discriminate closely related species such as cattle, yak, and buffalo; pig and goat (Chen, Liu, & Yao, 2010); cattle−buffalo and sheep−goat (Girish et al., 2005); swine and wild boar (Mutalib et al., 2012); and various fish species (Nebola, Borilova, & Kasalova, 2010). Kumar et al. (2014) developed RFLP

assay for the authentication of five most commonly used meat species namely cattle, buffalo, pig, sheep and goat. Two different REs (*Alu1* and *Taq1*) were used for the digestion of PCR products and distinctive digestion profiles allowed to differentiate each species. RFLP assays were also developed and applied on the PCR products of cat (Ali et al., 2015a), dog (Rahman et al., 2015b) and turtle (Asing et al., 2016b). Besides this method, an universal primers set was designed from the mitochondrial cytb gene for the amplification of 359 bp DNA fragments from six species including pig, beef, buffalo, goat, chicken, rabbit and quail. The species were discriminated from the restriction digestion pattern generated by the digestion of five Res such as *BsaJI, AluI, BstUI, MseI and RsaI* (Murugaiah et al., 2009). However, these methods are mostly based on single and long-length DNA targets which break down under natural or environmental decomposition and food processing treatments, making them less trustworthy and inconclusive for forensic investigation (Bottero & Dalmasso, 2011; Focke et al., 2010).

v ***Real-Time PCR***

In contrast to conventional PCR assays, real-time PCR techniques are especially promising since they are fast, automated, highly sensitive and offer both the detection and quantification opportunities of the analyte targets at real-time, eliminating the need of post PCR analysis such as time consuming electrophoresis (Asing et al., 2016a; Cheng et al., 2014). Moreover, multiplex quantitative PCR (mqPCR) might be highly advantageous over the singleplex qPCR methods because it could detect and quantify multiple target oligos in a single assay platform, saving both analytical cost and time (Ali et al., 2015d; Iwobi et al., 2015). Particularly, real-time PCR involves in the directly monitoring the generation of PCR products during each amplification cycle and able to measure at the exponential phase of the reaction there is no need to complete the reaction. Unlike end-point (conventional) PCR assay, this system allows quantifying the PCR products at an

initial stage of the reaction that is more precise and accurate. As fluorescent molecules are used to collect the real-time data, since there is high correlation between intensity of the fluorescent dye and the quantity of PCR products (Fajardo et al., 2010). Two general categories of fluorescent chemistries, namely, double-stranded (ds) DNA-intercalating dyes such as SYBR Green (Asing et al., 2016a) or Eva Green (Safdar & Abasıyanık, 2013) and probe based chemistry such as TaqMan (Ali et al., 2012) or Molecular Beacon (Hadjinicolaou, Demetriou, Emmanuel, Kakoyiannis, & Kostrikis, 2009) probes are available for the real-time PCR systems. The main drawback of the DNA-intercalating dye chemistry is that it non-specifically binds any dsDNA including primer-dimers that are available in the reaction tube, making the detection false positive and unreliable (Arya et al., 2014). Moreover, some dyes are known to inhibit the PCR reaction (Gudnason et al., 2007). In contrast, TaqMan probe based method is greatly promising since both the probe and primers find their appropriate partners in the template site, offering double checking opportunity that enhances assay specificity and reliability (Ali et al., 2012a). Because fluorescent signal is generated only when hybridize the specific probe due to the DNA polymerase moves by and cleaves off the probe's quencher molecule. In addition, TaqMan probe techniques are also suitable for the development of mqPCR systems because specific probes could be labeled with different reporter dyes that allow the identification of the amplified targets formed by one or multiple primer sets in a single PCR assay tube (Arya et al., 2014).

Several simplex and multiplex qPCR reports have been introduced for the identification and quantification species in food products. For example, a SYBR Green I oriented qPCR method was developed for the quantification of bovine milk adulteration in buffalo cheese products. The technique successfully identified the adulterated bovine milk in most of the marketed buffalo cheese samples (Lopparelli, 2007). SYBR Green fluorescence also used for the detection and quantification of bovine, porcine, caprine,

goose, turkey, chicken, and equine (Okuma & Hellberg, 2015); pork (Soares, Amaral, Oliveira, & Mafra, 2013) and box turtle (Asing et al., 2016a). On the other hand, Safdar et al. (2014) used the EvaGreen fluorescence dye, to develop a duplex qPCR assay for the reliable and rapid detection of bovine and caprine species in ruminant feeds. The method was optimized under heat treated (133^0 C and 3 bar for 20 min) bovine and caprine admixed meat. Safdar et al. (2013) (Safdar & Abasıyanık, 2013) also introduced another Eva Green approaches for the discrimination of beef and soybean in sausages. Iwobi et al. (2015) introduced TaqMan based m-qPCR assay for the quantification of beef and pork in minced meat. The sensitivity of the method was 20 genome equivalents and the validation of the method was carried out on various marketed minced meat products. Another TaqMan based mqPCR approach for the differentiation of bovine and buffalo in dairy samples. The method was validated by the analysis of commercial products with satisfactory results (Drummond et al., 2013). A TaqMan probe qPCR assay was reported for the authentication of species and gender origin of beef. This method consist of two reactions: bovine-specific qPCR and Y-chromosome–specific mqPCR. The technique is highly powerful tool for the discrimination of beef gender (Herrero et al., 2013). TaqMan probe based mqPCR also applied for the quantification of pork, beef, sheep and horse (Köppel, Ruf, & Rentsch, 2011); pork, duck, chicken, goose and turkey (Köppel, Daniels, Felderer, & Brünen-Nieweler, 2013); red deer, sika deer and fallow deer (Druml, Grandits, Mayer, Hochegger, & Cichna-Markl, 2015) and pig, chicken and duck (Cheng et al., 2014).

Although numerous mqPCR have been documented, but to the best of our knowledge, no mqPCR assays have been documented for the simultaneously detection and quantification of beef, buffalo and pork in food products.

2.3.3.2 Validation of PCR Method

(a) ***Definition***

According to Taverniers et al. (2004) "Validating a method is investigating whether the analytical purpose of the method is achieved, which is obtaining analytical results with an acceptable uncertainty level". Subsequently, Green (1996) depicted "Method validation is the process of proving that an analytical method is acceptable for its intended purpose". To fulfill this definition, the PCR method need to be properly optimized, standardized and developed so that it can be adapted to accomplish performance characteristics that are consistent with the purpose of the assay. (World Organization for Animal Health, 2009).

(b) ***Practical Evaluation of Parameters and Acceptance Criteria***

Various parameters of the PCR assay have to be tested to check the fitness of the method performance. A method can be accepted for routine analysis, if it complies with the predetermined criteria. During development and in-house validation of singleplex and multiplex PCR methods the following parameters need to be evaluated (Broeders et al., 2014).

i) Applicability

In the applicability statement, the developer should clearly describe the scope of the method with complete information, such as name of target species, which matrix is intended, or the amount of DNA have been analyzed. The method need to be assessed using several matrices namely, raw and processed materials, food and feed, and genomic DNA and plasmid DNA. Moreover, to detect the probable PCR inhibitors, different amounts of DNA can be analyzed. Reproducible results need to be produced for as many matrices as possible (Broeders et al., 2014).

ii) Practicability

To evaluate the practicability of the assay, blind samples need to be tested by the routine laboratory. Herein, new method can be run in ~~combined~~ combination with existing methods that had been already applied in the laboratory under the same conditions. To further evaluate the practicability, the developed method need to be transferred to a second laboratory to confirm the reproducible results (Broeders et al., 2014).

iii) Optimization and Standardization of Reagents and Determination of Critical Control Parameters

Collection and preparation of sample as well as DNA extraction procedures are all critical parameters in assay performance and should be optimized for good results. Appropriate DNA extraction methods vary depending on sample types. For example, extraction of DNA from raw meat samples is relatively easy, while that from complex matrices is more difficult. It is essential to develop an efficient and reproducible extraction method prior to perform further validation of the PCR assay. All apparatus used during validation process must be calibrated according proper protocols.

It is also important to determine the ability of the assay to remain unaffected due to slight variations in the main parameters during the development of the PCR method. To assess the critical parameters of the method it is essential to achieve an optimized PCR assay. Examples of such parameters include: concentration of $MgCl_2$, primers, buffer, dNTP and DNA Taq polymerase as well as annealing time and temperature. To identify the critical points that must be entirely be controlled in the assay, critical control parameters characterization is mandatory (Belak, & Thorén, 2004).

iv) Repeatability

Compliance between replicates within and between runs of the qPCR assay must be considered. This provides significant information about the method before further validation is performed. If excessive inconsistency is found, it should be perfected prior to ~~continue~~ the validation process. To check the PCR assay repeatability, each replicate should be considered as an independent sample. For example, for a replicate (e.g. a triplicate), three different aliquots of DNA extract are prepared for a specimen and amplified, and the variation from the mean value detected is determined as an indication of repeatability. Therefore, use of single DNA extract to analyze triplicate amplifications in not acceptable. Inter-run coefficient of variation of the qPCR assay can be determined by using the Ct-values generated from the replicated samples (Belak, & Thorén, 2004).

v) Determination of Analytical Specificity and Sensitivity

Specificity of the PCR assay is defined as the ability of the system to discriminate the target species from other non-target species. The specificity of the assay is determined by analyzing DNA extract from target and genetically related species. Allowable cross-reactivity is mainly dependent on the desired purpose of the assay and must be determined for each case.

Limit of detection (LOD) or sensitivity of the assay is defined as the lowest quantity of DNA detected by the assay. Serially diluted extracted DNA is used until the assay can no longer detect the target in question in more than 5% of the replicates to determine the assay's sensitivity (Belak, & Thorén, 2004).

vi) Establishing Reproducibility of the Assay

Reproducibility plays an important role to evaluate the assay precision. An identical method (reagents, protocol and controls) is applied in various laboratories to determine the assay reproducibility. At least three laboratories test results of the same set of specimens (minimum of 20 samples) with identical aliquots are required to validate the assay reproducibility as well as ruggedness of the assay (Belak, & Thorén, 2004).

For DNA-based procedures, the following additional information should be supplied in particular (Codex Alimentarius Commission, 2010):

Primer pairs

"General methods have to provide the defined primer pairs and the sequence they target. Recommendations as to the efficiency/use of primer set have to be clearly stated, including if the primers are suitable for screening and/or quantification".

Amplicon length

"Food processing will generally lead to a degradation of target DNA. The length of the amplified product may influence the PCR performance. Therefore the selection of shorter amplicon sizes (within reason) will increase the possibility to get a positive signal in the analysis of highly processed foodstuffs. In general the length of the amplified fragment for the taxon-specific DNA sequence and the target sequence should be in a similar size range".

Whether the method is instrument or chemistry specific

"At the moment a number of different types of real-time instruments and chemistries are available. These instruments and chemistries may have different performance such as

stability of reagents, heating and cooling characteristics, which affects ramp rates and affects the time necessary for a whole PCR run".

"Beside the differences in the heating and cooling system there are differences in the technique and software used to induce and subsequently to record the fluorescence. The detection and quantification of the fluorescence could also vary according to the recording instruments and software used. Qualitative methods generally tend to be less instrument-specific than quantitative methods".

"The methods are generally instrument and chemistries dependent and cannot be transferred to other equipment and chemistries without evaluation and/or modification".

CHAPTER 3: MATERIALS AND METHODS

3.1 Collection of Sample

Authentic fresh muscle of the beef (*Bos taurus*), buffalo (*Bubalus bubalis*), goat (*Capra hiscus*), lamb (*Ovis aries*), chicken (*Gallus gallus*), duck (*Anas platyrhychos*), pigeon (*Columba livia*), quail (*Coturnix coturnix*)) and selected aquatic species such as cod (*Gadus morhua*), salmon (*Salmo salar*), pangas (*Pangasius pangasius*), tuna (*Thunnus orientalis*), tilapia (*Oreochromis niloticus*) and rohu (*Labeo rohita*), frog (*Rana kunyuensis*) and turtle (*Cuora amboinensis*)), as well as five plant species such as wheat (*Triticum aestivum*), onion (*Allium cepa*), garlic (*Allium sativum*), ginger (*Zingiber officinale*) and pepper (*Capsicum annuum*) were purchased in triplicate on three different days from various wet markets and supermarkets (Pasar Borong Selangor, Serdang, Pudu Wet Market, Kuala Lumpur and Tesco, Petaling Jaya, Selangor). Pork (*Sus scrofa*) was purchased in triplicates from three different vendors from a Chinese wet market in Sri Kambangan, Selangor, Malaysia. Meat from three different euthanized dogs (*Canis lupus familiaris*), cats (*Felis catus*) and rats (*Rattus rattus*) were collected from Dewan Bandaraya Kuala Lumpur (DBKL) as described elsewhere (Ali, et al., 2015d). Monkey (*Macaca fascicularis*) meat was obtained from the Department of Wildlife and National Parks (DWNP) Peninsular Malaysia (Cheras, Kuala Lumpur) as described elsewhere (Rashid et al., 2015b). Commercial beef and pork frankfurters, meatballs and burger of different brands were purchased in triplicates from different stores in Kuala Lumpur, Malaysia. In addition, seven curry samples were purchased from seven different restaurants. Transportation of all samples and products was carried out under ice-chilled condition (4^0C) and were stored at -20^0C until further use to prevent the natural and enzymatic degradation of DNA.

3.2 Extraction of DNA

Yeastern Genomic DNA Mini Kit (Yeastern Biotech Co., Ltd, Taipei, Taiwan) was used for the extraction of total DNA from meat and fish samples (Rashid et al., 2015b). Briefly, 20 mg of muscle tissues was ground and homogenized with a micro pestle followed by the addition of lysis buffer and proteinase K. The mixture was incubated at 60^0 C for cell lysis and protein degradation. The spin column was used for the binding of DNA to the glass fibre matrix under centrifugation. Ethanol containing wash buffer was used to remove any contaminants. The purified DNA was eluted in elution buffer. DNA of Plant species (wheat, onion, garlic, ginger and pepper) was extracted by using the DNeasy Plant Mini Kit (QIAGEN GmgH, Hilden, Germany). NucleoSpin Food DNA kit (MACHEREY-NAGEL GmbH & Co. KG, Duren, Germany) was used for the extraction of DNA from food products (frankfurters, meatballs and burgers) (Hird, Lloyd, Goodier, Brown, & Reece, 2003). Concentration and purity of the extracted DNA were checked using UV–VIS Spectrophotometer (NanoPhotometer Pearl, Implen GmbH, Germany) based on the absorbance at 260 nm and ratio at A_{260}/A_{280}, respectively (Napolitano et al., 2014).

3.3 Development of Biomarkers for Multiplex PCR

Proper design of primers is a vital step/factor for an efficient and successful PCR amplification. Higher efficiency and maximum specificity of PCR depends on the optimal matching of primer sequences and also adequate primer concentrations (He, Marjamäki, Soini, Mertsola, & Viljanen, 1994). An inaccurately designed primers may lead to little product or formation of primer-dimer and/or non-specific products (Abd-Elsalam, 2003). The development of multiplex PCR primer sets is more complex and complicated because all primers are annealed to their respective targets under a single set of PCR conditions. Specificity and Tm are also more important in a multiplex system over the conventional PCR (Razzak, 2015). In addition, PCR products length (amplicon size) should also be

taken in account during the design of primers. The size of the amplicons depends on the resolution capability of the detection system; so that the generated PCR products can distinguish easily from one another.

In order to design the primers, whole genomic sequences of the target species were retrieved from NCBI database (http://www.ncbi.nlm.nih.gov/) and were aligned using Molecular Evolutionary Genetics Analysis version 5 (MEGA5) alignment tool (Tamura et al., 2011) for identifying the inter-species hyper-variable and intra-species conserved regions. A publicly available primer designing software, Primer3Plus was used to get the desired sequences of the primers.

The following criteria and guidelines were considered for the design of species specific primers for amplifying specific target sequence:

3.3.1 Primer Length

The length of primer plays an important role for the specificity as well as annealing time and temperature for the target binding; these parameters are vital for a successful PCR (Wu, Ugozzoli, Pal, Qian, & Wallace, 1991). Too long primers may decrease the efficiency of template DNA binding at normal annealing temperature due to the chance of forming secondary structure; whereas, too short primers may result in low specificity and non-specific amplification (Abd-Elsalam, 2003). The ideal primer length should be 18-28 nucleotides but usually good activity is obtained with primers having 20-24 nucleotides in length (Dieffenbach, Lowe, & Dveksler, 1993).

3.3.2 GC Content

One of the most important characteristics of primer is its GC content which refers to its annealing strength. To get good PCR product, a reasonable GC content should be maintained. Tm and annealing temperature (Ta) fully depend on the percentage (%) of

GC content (Rychlik, Spencer, & Rhoads, 1990). The ideal GC content is 40-60% and 3 or more G's or C's at the 3′-end should be avoided because it has adverse effect to the primer specificity. Primer should not have long polyG or polyC stretches which result in non-specific annealing (Ali, Razzak, & Hamid, 2014b).

3.3.3 Melting and Annealing Temperature

Melting temperature (Tm) is an important parameter of primer, since it plays a vital role for primer annealing. Primers with Tm of 55-65^0 C work best in most of amplification reactions. Tm of both forward and reverse primers should have similar as they are annealed simultaneously. Moreover, multiplex PCR efficiency is effected by a little differences of Tm between the primer sets. Since all targets are amplified in a multiplex PCR in a single reaction mixture, all primers should have very close Tm. The acceptable Tm variation is 3-5^0 C between the primers but to get good result ≤ 2 °C Tm variation is preferable. Significantly lower Tm of a primer than the PCR annealing temperature (Ta) may cause failure to anneal and extend, while significant higher Tm may lead to non-hybridization and can extend at an incorrect location along the DNA sequence (Ali et al., 2014b). The approximate Tm value of the primer can be calculated by using the formula (generally valid for oligos in the 18–30 base range) of Wallace et al. (1979) Tm (°C) = 2 x (nA+nT) + 4 x (nG + nC), where, nA, nT, nG and nC are the number of respective nucleotides in the primer. Mismatching between template DNA and primers is the main feature for specificity and Tm of the designed primers. Because the presence of 1% of base mismatch in the double-stranded (ds) DNA would reduce the Tm value by 1-1.5^{0}C (Matsunaga et al., 1999). However, the increasing of percent of mismatching with non-target species leads to the decrease of Tm value, but higher specificity. On the other hand, the Tm of the TaqMan probes of the real-time PCR must be 8-10^{0}C degree higher than that of primers to facilitate the preferential binding of the probes prior to the annealing of the primers to the template (Arya et al., 2005).

3.3.4 3'-end Specificity

For the design of primer to achieve a successful PCR experiment, 3′-end sequence is very important because during the extension step, DNA polymerase starts to attach nucleotides from the 3′-end of a primer. Since, complete annealing of the primer 3′-end is mandatory and incomplete binding at the 3′-end results in lower PCR or often no PCR products (Yuryev, 2007). Therefore, primers should have mismatch with non-target species at 3′-end, as it prohibits the PCR amplification (Ali et al., 2014b). It is well known that for the control of mis-priming, the 3′-end position of the primer plays an important role (Kwok et al., 1990).

3.3.5 Primer-Primer Interactions

Primer should have a minimum of intermolecular or intramolecular homology that can promote to the formation of either primer dimerization or hairpins (Figure 3.1a & b). Primer with nucleotide sequences that would allow anneal one primer to other primer(s), results in primer-dimer formation (Figure 3.1a), particularly when 3′-ends of the primers anneal to each other. Inter primer homology in the middle position of two primers may also interfere with hybridization. Primer with a self-homology region result in "sanp back" or able to form partially double stranded structures, hairpin (Figure 3.1b), which will interfere with annealing to the template. To overcome the formation of hairpin, it is recommended that intra-primer homologies of 3 bp or more should be avoided (Abd-Elsalam, 2003).

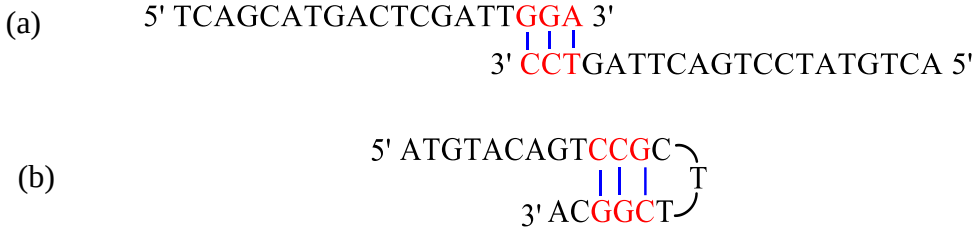

Figure 3.1: Primer-Primer interactions (a) primer-dimer; (b) hairpin (Adapted from Ali et al., 2014b).

3.3.6 Specificity

Primer specificity is checked in three different ways. At first, primers were aligned by using online Basic Local Alignment Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to screen the identical and distant species. Secondly, to determine the total number of mismatch between target and non-target species, the primers are multiple sequence aligned with some common species using an alignment tool such as ClustalW (http://www.genome.jp/tools/clustalw/) or MEGA5. Finally, each primer is assayed in PCR experiment with template DNA of non-target species to confirm the specificity.

3.3.7 Design of Species-Specific Primers and Probes

Six sets of primers were designed targeting mitochondrial cytb and ND5 genes because of their higher degree of divergence. This results in sufficient conserved regions within the same species but adequate polymorphism among the closely related species (Mohamad et al., 2013; Razzak et al., 2015). The Sequences of cytb and ND5 genes of cow (V00654.1), buffalo (NC_006295.1) and pig (AF034253.1) species were retrieved from the National Centre of Biotechnology Information (NCBI) database and were aligned using the MEGA5 alignment tool (http://www.megasoftware.net/) to identify the hyper-variable and conserved regions. Publicly available software Primer3Plus (version: 2.4.1) (http://www.bioinformatics.nl/cgibin/ primer3plus/ primer3plus.cgi) was used to design the six sets (two sets for each species (one set from cytb and another set from ND5)) of primers (Table 3.1). The initial specificity of the designed primers was tested by screening with nucleic acid sequences of similar and distinct species using online Basic Local Alignment Tool (BLAST) in NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to avoid cross-species amplification. To determine the total mismatch between the target and non-target species, the primers were in-silico screened with 17 animal species, namely, cow (*Bos taurus*), buffalo (*Bubalus*

bubalis), pig (*Sus scrofa*), horse (*Equus caballus*), goat (*Capra hircus*), deer (*Cervus nippon*), sheep (*Ovis aries*), donkey (*Equus africanus*), dog (*Canis lupus*), rabbit (*Oryctolagus cuniculus*), monkey (*Macaca fascicularis*), cat (*Felis catus*), chicken (*Gallus gallus*), duck (*Anas platyrhynchos*), pigeon (*Columba livia*), quail (*Coturnix coturnix*) and rat (*Rattus norvegicus*), 8 aquatic species, namely, cod (*Gadus morhua*), salmon (*Salmo salar*), tuna (*Thunnus orientalis*), tilapia (*Oreochromis niloticus*), rohu (*Labeo rohita*), Pangas (*Pangasius pangasius*), frog (*Rana kunyuensis*) and turtle (*Cuora amboinensis*) and four plant species, namely, wheat (*Triticum aestivum*), onion (*Allium cepa*), ginger (*Zingiber officinale*) and pepper (*Capsicum annuum*) using a ClustalW multiple sequence alignment program (http://www.genome.jp/tools/clustalw/) and MEGA5 alignment tool. The final specificity was confirmed through a PCR assay against templates of 25 alien species. After conformation of the primer sets, three probes for the real-time PCR assay also designed based on the primer set of ND5 gene of cow and cytb gene of buffalo and pig species by using Primer3Plus software and initial specificity were tested by using BLAST and probe sequences were listed on Table 3.1. The primers and probes were synthesized by Integrated DNA Technologies (IDT), Singapore and supplied by First BASE Laboratories Sdn Bhd., Selangor, Malaysia.

Table 3.1: Name and sequence of primers and probes used in this study.

Name	Species	Target gene	Sequence (5′ - 3′)	Tm	Amplicon size (bp)
Cocytb	Cow (*Bos taurus*)	Cytb	Forward: GGAGTACTAGCCCTAGCCTTCTC Reverse: CTACTAGGGCTCAGAATAGGCATT	57.8 58.7	120
CoND5	Cow (*Bos taurus*)	ND5	Forward: GGTTTCATTTTAGCAATAGCATGG Reverse: GTCCAATCAAGGGTATGTTTGAG Probe: ACAAATCTCAATACCTGAGACCTCCAACAGA	61 59.8 68.5	106
Bucytb	Buffalo (*Bubalus bubalis*)	Cytb	Forward: GGGTTCTAGCCCTAGTTCTCTCT Reverse: ATGGCCGGAACATCATACTT Probe: AATCCTCATTCTCATGCCCCTGCTACA	58.6 59.3 70.3	90
BuND5	Buffalo (*Bubalus bubalis*)	ND5	Forward: TCGCCTAGCTTCTTACACAAAC Reverse: TGGTTTGTGACTGTGATGGAT	58.7 58.8	138
Pocytb	Pork (*Sus scrofa*)	Cytb	Forward: TATCCCTTATATCGGAACAGACCTC Reverse: GCAGGAATAGGAGATGTACGG Probe: CCTGCCATTCATCATTACCGCCC	60.9 58.7 70.7	146
PoND5	Pork (*Sus scrofa*)	ND5	Forward: GATTCCTAACCCACTCAAACG Reverse: GGTATGTTTGGGCATTCATTG	58.6 60.1	73

3.3.8 Construction of Pairwise Distance and Phylogenetic Tree

The pairwise distance and phylogenetic tree were constructed by aligning the each amplicon sequence with the respective gene sequences of target and 28 non-target species using the neighbour-joining method of MEGA5 version 5.1 software (Tamura et al., 2011). For example, the sequence of beef cytb amplicon was aligned along with the cytb gene of beef and other 28 non-target species such as buffalo, goat, sheep, deer, donkey, horse, pork, dog, cat, rabbit, monkey, chicken, duck, pigeon, quail, rat, salmon, tuna, cord, tilapia, rohu, pangas, frog, turtle, wheat, onion, ginger, chili. Pairwise distance of other five amplicons were constructed in the same way.

3.3.9 Construction of 3D Plots

3D plot of each primer set was generated from three variables such as forward and reversed primer mismatch (Section 3.3.7) and pairwise distance (Section 3.3.8) data of individual primer sets using XLSTAT2014 version 2014.5 software (Addinsoft, 2013).

3.4 Development of Simplex PCR Assay

3.4.1 Optimization of Simplex PCR Assay

Simplex PCR of individual primer was developed using DNA extracted from muscle tissue of relevant species (cow, buffalo and pig). Total volume of all amplification assays were 25 µL comprised of 5 µL of 5X GoTaq Flexi Buffer, 0.2 mM each of dNTP, 2.5 mM $MgCl_2$, 0.625 U GoTaq Flexi DNA Polymerase (Promega, Madison, USA), 0.4 µM of each primer (Table 3.2) and 2 µL (20 ng/µL) of the total DNA template. Negative control (PCR amplification without template DNA) was carried out for each PCR reaction to avoid any contamination with PCR mixture. In the simplex PCR specificity test, I also used 0.4 µL a universal eukaryotic primer (forward primer: 5' AGGATCCATTGG AGGGCAAGT 3' and reverse primer: 5' TCCAACTACGAGCTTTTTAACTGCA 3') of 99 bp amplicon sized from 18S rRNA gene (Safdar & Junejo, 2015). ABI 96 Well Verity

Thermal Cycler (Applied Biosystems, Foster City, CA, USA) was used for the PCR reaction following the cycling parameters of an initial denaturation at 95^0C for 3 min followed by 35 cycles of denaturation at 95^0C for 30s, annealing at 58-62^0C for 30 – 35 s, extension at 72^0C for 40 s and the final extension at 72^0C for 5 min (Table 3.3). PCR products were kept at -20^0C for further analysis.

Table 3.2: Concentration of simplex PCR components.

Primer	dNTP (mM)	$MgCl_2$ (mM)	Taq pol (unit)	Primer (µM)
Cocytb	0.20	2.50	0.625	0.40
CoND5	0.20	2.50	0.625	0.40
Bucytb	0.20	2.50	0.625	0.40
BuND5	0.20	2.50	0.625	0.40
Pocytb	0.20	2.50	0.625	0.40
PoND5	0.20	2.50	0.625	0.40

Note: 5 µl of 5X GoTaq Flexi Buffer was used in all PCR experiments.

Table 3.3: Cycling parameters of simplex PCR reactions.

PCR reaction	Initial denaturation	35 cycles			Final extension
		Denaturation	Anneling	Extension	
Cocytb	95^0 C for 3 min	95^0 C for 30 s	60^0 C for 35 s	72^0 C for 40 s	72^0 C for 5 min
CoND5	95^0 C for 3 min	95^0 C for 30 s	60^0 C for 30 s	72^0 C for 40 s	72^0 C for 5 min
Bucytb	95^0 C for 3 min	95^0 C for 30 s	60^0 C for 30 s	72^0 C for 40 s	72^0 C for 5 min
BuND5	95^0 C for 3 min	95^0 C for 30 s	60^0 C for 30 s	72^0 C for 40 s	72^0 C for 5 min
Pocytb	95^0 C for 3 min	95^0 C for 30 s	60^0 C for 35 s	72^0 C for 40 s	72^0 C for 5 min
PoND5	95^0 C for 3 min	95^0 C for 30 s	60^0 C for 35 s	72^0 C for 40 s	72^0 C for 5 min

3.4.2 Gel Electrophoresis

In order to detect of species-specific simplex PCR amplified products, DNA visualization was accomplished by conventional gel electrophoresis and finally on automatic QIAxcel Advanced Capillary Electrophoresis System.

3.4.2.1 Conventional Gel Electrophoresis

To perform the conventional gel electrophoresis, 2% (w/v) agarose gel was prepared as follows:

Firstly, 150 ml of 1 X Tris-borate-EDTA (TBE) buffer was taken in a 250 ml beaker subsequently added 3 g of agarose in the buffer and mixed well followed by heating in a microwave oven to dissolve completely. Once the gel temperature reached 50-60^{0}C, 5-6 µL of fluorosafe DNA stain (1st Base Laboratories, Selangor, Malaysia) was added and mixed gently. Then, the molten gel mixture was poured into a horizontal electrophoresis tray containing the well comb. The gel then solidified for 20-30 minutes. After placing the gel tray inside the 1× TBE buffer containing tank, 6 µL PCR products and 100 bp DNA ladder (Promega, USA) were loaded into the gel wells. After that, the gel electrophoresis (SUB13, Hoefer, Inc., California, USA) was carried out at 120 volts for about 70 min resulting the PCR products were separated on the basis of molecular size. Finally, the PCR products banding profile was visualized under a gel image documentation system (AlphaImager HP, Alpha Innotech Corp., California, USA).

3.4.2.2 QIAxcel Advanced Capillary Electrophoresis System

The QIAxcel Advanced Capillary Electrophoresis System offer rapid, fully automatic, very sensitive and high resolution (can separate the products with 3-5 bp differences), required low amount of sample and convenience due to the use of ready-to-use gel cartridge. Due to the automatic system, minimum handling interaction is required for samples analysis resulting in little manual error and excluding the laborious gel preparation. The system is able to generate both gel images and electropherograms of the analyzed samples in a single analysis platform by applying the electrical current to a gel-filled capillary cartridge via individual electrode of each capillary. Unlike conventional

gel electrophoresis, electropherograms of the PCR products can determine the accurate sizes of the amplicons.

3.4.3 Specificity Test of Simplex PCR Assay

Specificity of the simplex PCR assays were analyzed by cross-amplification with the extracted DNA of three targets species (cow, buffalo and pig), 20 non-target of terrestrial and aquatic animal species (goat, lamb, dog, cat, rabbit, monkey, donkey, chicken, duck, pigeon, quail, rat, salmon, tuna, cod, tilapia, rohu, pangas, frog and turtle) and 5 plant species (wheat, onion, garlic, ginger and pepper) which are commonly used in food products. In the simplex PCR specificity test, I also used 0.4 µL a universal eukaryotic primer (forward primer: 5' AGGATCCATTGGAGGGCAAGT 3' and reverse primer: 5' TCCAACTACGAGCTTTTTAACTGCA 3') of 99 bp amplicon sized from 18S rRNA gene (Safdar & Junejo, 2015).

3.4.4 PCR Product Sequencing

Extracted DNA of three target species (cow, buffalo and pig) were amplified using specific primer set and were confirmed the successful amplification with DNA visualization by gel electrophoresis. The amplified products were sequenced after cloning into the pJet1.2 blunted Vector by Integrated DNA Technologies (IDT), Singapore and supplied by First BASE Laboratories Sdn Bhd., Selangor, Malaysia. Briefly, the blunt-end of the purified PCR products constructed by proofreading DNA polymerases ligated into the cloning site of pJet1.2 blunted Vector followed by introducing the recombinant plasmid into living *E. coli* cells. The lethal gene of the vector is disrupted by the insertion of PCR product facilitating the propagation of only recombinant plasmid containing bacterial cells since plasmid contains *in vitro* transcription promoter T7. A single transformation colony of the recombinant plasmid containing cells is produced due to the expression of ampicillin-resistance gene which is encoded in the plasmid. After

purification of the recombinant plasmid containing insert was separated by digestion with restriction enzyme. Finally, the PCR products were sequenced to determine the original order of the nucleotides the products.

The derived sequences were then compared with GenBank sequences using the nucleotide basic local alignment search tool (BLAST) to evaluate any species match and were also aligned with specific gene sequence using MEGA5 software to determine the similarity with specific species.

3.5 Development of Tetraplex PCR Assay

I developed double genes targeted tetraplex PCR assay for cow and buffalo prior to develop hexaplex PCR for cow, buffalo and pig.

3.5.1 Optimization of Tetraplex PCR Assay

To develope a tetraplex PCR system for the simultaneous amplification of four targeted genes, two genes (cytb and ND5) of each target species (cow and buffalo) were initially optimized in two duplex platforms for the primers of Cocytb and Bucytb as well as for CoND5 and BuND5 followed by triplex PCR of Cocytb, CoND5 and BuND5 and finally multiplex PCR of Cocytb, Bucytb, CoND5 and BuND5. All amplifications were performed in a total volume of 25 µL containing of 5 µL of 5X GoTaq Flexi Buffer in Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The concentration of primers and other reagents and the cycling parameters were given in Table 3.4 and 3.5, respectively. As in simplex PCR negative control was also performed in all multiplex PCR reactions to eliminate any PCR mixture contamination. Due to the poor resolution of agarose gel, the PCR products were analysed using QIAxel DNA High Resolution Kit (QIAGEN GmbH, Germany) with size marker containing 500, 400, 300, 250, 200, 150, 100, 75, 50 and 25-bp marker DNA and with 600 and 15-bp alignment marker in QIAxcel

Advanced Capillary Electrophoresis System (QIAGEN GmbH, Hilden, Germany) for both gel image and electroferogram.

Table 3.4: Concentration of PCR components of various PCR assays.

PCR	dNTP (mM)	$MgCl_2$ (mM)	Taq pol (unit)	Primer (µM)
Duplex and Triplex	0.2	2.5	0.94	0.2 – 0.4
Tetraplex	0.25	3.5	1.0	0.16 – 0.4

Note: 5 µl of 5X GoTaq Flexi Buffer was used in all PCR experiments.

Table 3.5: Cycling parameters of various PCR reactions.

PCR reaction	Initial denaturation	35 cycles			Final extension
		Denaturation	Anneling	Extension	
Duplex and Triplex	95^0 C for 3 min	95^0 C for 30 s	60^0 C for 45 s	72^0 C for 45 s	72^0 C for 5 min
Tetraplex	95^0 C for 3 min	95^0 C for 40 s	60^0 C for 60 s	72^0 C for 50 s	72^0 C for 5 min

3.5.2 Specificity Test of Tetraplex PCR Assay

Specificity of the tetraplex assay was analyzed by cross-amplification with the extracted DNA of 20 non-target of terrestrial and aquatic animal species (goat, lamb, dog, cat, rabbit, monkey, donkey, chicken, duck, pigeon, quail, rat, salmon, tuna, cod, tilapia, rohu, pangas, frog and turtle) and 5 plant species (wheat, onion, garlic, ginger and pepper) which are commonly used as food matrices.

3.5.3 Limit of detection (LOD) of Tetraplex PCR Assay under Raw State

To determine the limit of detection (LOD) of the tetraplex and mPCR assay, extracted DNA of target-species (cow, buffalo and pig) were diluted serially from higher concentration (50 ng/μL) to 10.0, 5.0, 1.0, 0.5, 0.2, 0.1, 0.05, 0.02 and 0.01 ng/μL) and was used as a template.

3.5.4 Sensitivity test of Tetraplex PCR Assay under Binary Meat Admixture

To evaluate the tetraplex PCR assay sensitivity a binary admixture of beef and buffalo meat was prepared. Firstly, both raw beef and buffalo meat were minced and blended separately followed by mixing of beef and buffalo meat in the ratios of 99:1, 95:5, 90:10, 75:25, 50:50, 25:75, 10:90, 5:95 and 1:99 in a 100 g specimens (Rea et al., 2002). The prepared admixtures were homogenized by vigorous blending.

3.5.5 Sensitivity test of Tetraplex PCR Assay under Commercial Product (Burger)

To simulate the adulteration effect on commercial meat products, different types of burgers were prepared according to Ali et al. (2012b). At first pure beef and buffalo burgers were prepared separately and then the prepared beef burgers were spiked with 1% buffalo meat and buffalo burgers were spiked with 1% beef. A third category burgers were prepared by spiking of 1% buffalo meat with commercial beef burgers. Typically, each type of burger was made by adding the following ingredients: 500 g of minced meat, 1 g of egg, 1 g of chopped onion, one teaspoon of cumin seed, ¼ teaspoon of cayenne pepper and 6 g of finely chopped sun-dried tomato and mixed well. Finally, the prepared mixture was equally divided into four parts and each part was pressed into the shape of a burger. The prepared burger was placed in a fridge for 1 h to firm up. The model and commercial burgers were treated to adjust the excessive cooking and boiling effects under autoclaving at 121^0 C and 15-psi pressure for 2.5 h. All samples were prepared in triplicate on three different days and were stored at -20^0 C until the DNA was extracted.

3.5.6 Target DNA Stability Test under Tetraplex PCR Assay

To mimic the usual cooking conditions, the raw meat samples were subjected to three different heat treatments. The first one was boiling, in which the beef and buffalo meat were boiled in water at 98^0 C on a hot plate for 45 min (Ali et al., 2015b). To simulate a conventional canning process and steam cooking practices, the meat samples were autoclaved at 121^0 C under 15-psi pressure for 2.5 h (Ali et al., 2015d). Further, the meat samples were subjected to microwave cooking which is a quick and modern system of heating at 500 watt, 600 watt and 700 watt for 30 min (Table 3.6) (Ali et al., 2015c). All treated samples were kept at -20^0 C for next uses.

Table 3.6: Different thermal processes applied to target meat samples.

Heat Treatment	**Condition**	**Time (min)**	**Pressure (psi)**	**References**
Boiling	98° C	45	-	Ali et al., 2015b
Autoclave	121° C	150	15	Ali et al., 2015d
Microwave	500, 600, 700 W	30	-	Ali et al., 2015b

3.6 Development of Hexaplex (Multiplex) PCR of Cow, Buffalo and Pig

After development of tetraplex PCR system, hexaplex (multiplex) PCR assay for the simultaneous amplification of six targeted genes, two genes (cytb and ND5) of each target species (cow, buffalo and pig) was optimized with the addition of two pig genes, Pocytb and PoND5.

3.6.1 Optimization of Multiplex (Hexaplex) PCR (mPCR) Assay

The mPCR assay was optimized in a 25 µL of total reaction volume comprised of 5 µL of 5X GoTaq Flexi Buffer, 0.25 mM each of dNTP, 4.0 mM $MgCl_2$, 1.25 U GoTaq Flexi DNA Polymerase (Promega, Madison, USA), 0.12-0.60 µM of each primer (Table 3.7) and 1 µL (20 ng/µL) of the total DNA template of each species and required quantity

of the nuclease free water. ABI 96 Well Verity Thermal Cycler (Applied Biosystems, Foster City, CA, USA) was used for the PCR reaction following the cycling parameters of an initial denaturation at 95^0 C for 5 min followed by 40 cycles of denaturation at 95^0 C for 50 s, annealing at 60^0 C for 90 s, extension at 72^0 C for 50 s and the final extension at 72^0 C for 7 min. For the detection of species-specific simplex PCR amplified products, DNA visualization was accomplished on QIAxcel Advanced Capillary Electrophoresis System (QIAGEN Hilden, Germany) for both gel image and electroferogram. PCR products were kept at -20^0 C for further analysis.

Table 3.7: Concentration of the primers used in mPCR assay.

Primer	**Cocytb**	**CoND5**	**Bucytb**	**BuND5**	**Pocytb**	**PoND5**
Concentration (μM)	0.60	0.40	0.12	0.20	0.20	0.50

3.6.2 Specificity Test of Multiplex PCR Assay

Specificity of the mPCR (hexaplex) assay was also analyzed by cross-amplification with the extracted DNA of 20 non-target of terrestrial and aquatic animal species and 5 plant species described in section 3.5.2.

3.6.3 Limit of Detection (LOD) of Multiplex PCR Assay under Raw State

To determine the limit of detection (LOD) of the mPCR assay, extracted DNA of target-species (cow, buffalo and pig) were diluted serially from higher concentration (50 ng/μL) to 10.0, 5.0, 1.0, 0.5, 0.2, 0.1, 0.05, 0.02 and 0.01 ng/μL) and was used as a template.

3.6.4 Sensitivity Test of Multiplex PCR Assay under Commercial Products (Frankfurters and Meatballs)

For the screening of commercial products using developed mPCR system, three types of ready-to-eat model meatballs and frankfurters of beef, buffalo and pork were prepared as per Razzak et al. (2015) (Table 3.8). The prepared beef, buffalo and pork products were deliberately adulterated by spiking of 1%, 0.5% and 0.1% of beef, buffalo and pork into a balanced amount of buffalo and pork; beef and pork; and beef and buffalo meat, respectively. Thus, prepared 0.1% contaminated frankfurters and meatballs were autoclaved at 121^0 C for 2.5 h under 15-psi and 45-psi pressure, respectively (Rahman et al., 2014). All samples were stored at -20^0 C until DNA was extracted.

Table 3.8: Formulation of model meatball and Frankfurter.

Ingredients	Meatball (~35 g/piece)			Frankfurter (~70 g/piece)		
	Beef	Buffalo	Pork	Beef	Buffalo	Pork
Minced meat	23[a]	23[a]	23[a]	45[a]	45[a]	45[a]
Soy protein	3	3	3	7.5	7.5	7.5
Starch/breadcrumb	5	5	5	6.5	6.5	6.5
Chopped onion	1	1	1	2.5	2.5	2.5
Chopped ginger	0.1	0.1	0.1	0.15	0.15	0.15
Cumin powder	0.75	0.75	0.75	0.75	0.75	0.75
Garlic power	0.5	0.5	0.5	0.5	0.5	0.5
Black pepper	0.14	0.14	0.14	0.23	0.23	0.23
Tomato paste	1.5	1.5	1.5	2.0	2.0	2.0
Butter	1.5	1.5	1.5	2.5	2.5	2.5
Salt	SA	SA	SA	SA	SA	SA
Others[b]	SA	SA	SA	SA	SA	SA

Note: [a]1%, 0.5% and 0.1% of beef, buffalo and pork meat were mixed with a balanced amount of respective minced meat to prepare ~35 g meatball and ~70 g frankfurter specimen. [b]Flavouring agents and enhancers. SA, suitable amounts.

3.6.5 Target DNA Stability Test under Multiplex PCR Assay

To check the stability of the developed mPCR assay, the raw meat samples were subjected to three different heat treatments. The first one was boiling, in which the beef, buffalo and pork meat were boiled in water at 98^0 C on a hot plate for 90 min (Ali et al., 2015b). To simulate a conventional canning process and steam cooking practices, the meat samples were autoclaved at 121^0 C under 45-psi pressure for 20 min and 2.5 h (Ali et al., 2015d). Further, the meat samples were subjected to microwave cooking which is a quick and modern system of heating at 500 watt, 600 watt and 700 watt for 30 min (Ali et al., 2015c). All treated samples were kept at -20^0 C for next uses.

3.7 Enzymatic Digestion and RFLP Analysis

The sequences of the amplified PCR products were retrieved from NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genbank) and a publicly available NEBcutter version 2.0 software (http://tools.neb.com/ NEBcutter) was used to select the specific and appropriate restriction endonucleases for all the PCR amplicons prior to test the mPCR-RFLP assay to ensure distinctive RFLP patterns for all targets. The restriction patterns of the PCR amplicons of beef, buffalo, and pork mitochondrial cytb and ND5 genes are given in Table 3.9.

Table 3.9: Restriction digests of the PCR products.

Target	Restriction enzyme	Amplicon size (bp)	Fragment size (bp)
Cocytb	*Eci*I	120	75, 45
CoND5	*Fat*I	106	87, 19
Bucytb	*Fat*I	90	50, 40
BuND5	*Alu*I	138	130, 8
Pocytb	*CviKI*-1	146	80, 45, 21
PoND5	*Fat*I	73	52, 21

3.7.1 Analysis of Beef and Buffalo PCR Products

The four PCR products of cow and buffalo were digested in two steps: firstly, four simplex PCR products were digested individually with appropriate restriction enzyme (Table 3.9) and then tetraplex PCR products of that species were digested using the same restriction enzyme and reaction conditions.

3.7.1.1 Digestion of Beef and Buffalo Simplex PCR Products

The simplex PCR products of beef cytb and buffalo ND5 genes were digested with EciI and AluI restriction endonucleases (New England Biolab, Ipswich, MA, United States), respectively. On the other hand, beef ND5 and buffalo cytb products were digested with FatI. The total volume of each digestion reaction was 25 µL, which was composed of 1 µg of unpurified PCR product, 1× digestion buffer (supplied with the enzyme), 1U of each enzyme, and a balanced amount of sterilized distilled water. The reaction mixtures were gently mixed and spun down and incubated at 37 °C with *EciI* and *AluI* and 55 °C with *FatI* in a shaking water bath for 60 min. Finally, the digestion reaction was stopped by heating the reaction mixtures at 65 °C for *EciI* and 80 °C for *AluI* and *FatI* for 20 min (Table 3.10).

Table 3.10: Restriction enzyme reaction conditions for the digestion of beef and buffalo simplex PCR products.

Target	Restriction enzyme	Amount of PCR Products (µg)	Incubation temp. and time	Deactivation temp. and time
Cocytb	*Eci*I	1.0	37 °C for 60 min	65 °C for 20 min
CoND5	*Fat*I	1.0	55 °C for 60 min	80 °C for 20 min
Bucytb	*Fat*I	1.0	55 °C for 60 min	80 °C for 20 min
BuND5	*Alu*I	1.0	37 °C for 60 min	80 °C for 20 min

3.7.1.2 Digestion of Beef and Buffalo Tetraplex PCR Products

The tetraplex PCR products of Cocytb, CoND5, Bucytb, and BuND5 were digested simultaneously in a 25 µL reaction mixture containing 16 µL of unpurified PCR product, 2.5 µL of digestion buffer, 1.5 µL of AluI, 2.5 µL of EciI, and 2.5 µL of FatI. The reaction was mixed by gentle shaking, spun down, and incubated in a shaking water bath first at 37°C for 60 min and then at 55°C for 60 min. Enzymatic digestion was stopped by heating the mixture at 80°C for 20 min in a water bath. The digests were separated in an automated QIAxcel Advanced Capillary Electrophoresis System (QIAGEN GmbH, Hilden, Germany) using a QIAxel DNA High- Resolution Kit (QIAGEN GmbH, Hilden, Germany).

3.7.2 RFLP Analysis of Simplex Pork PCR Products

Pork Pocytb and PoND5 PCR products were digested with CviKI-1 and FatI restriction endonucleases (New England Biolab, Ipswich, MA, United States) in a separate reaction tube of 25 µL reaction volume comprising 1 µg of unpurified PCR product, 1× digestion buffer supplied with the enzyme, 1U of each enzyme, and a required amount of sterilized distilled water. The reaction mixtures were mixed gently and spun down followed by incubation at 37°C for CviKI-1 and 55°C for FatI in a shaking water bath for 60 min to digest the targets properly. Post digested reaction was inactivated by heating the mixtures for 20 min at 80 °C for FatI while no inactivation was required for CviKI-1 enzyme (Table 3.11).

Table 3.11: Restriction enzyme reaction conditions for the digestion of pork simplex PCR products.

Target	Restriction enzyme	Amount of PCR Products (µg)	Incubation temp. and time	Deactivation temp. and time
Pocytb	*CviKI*-1	1.0	37°C for 60 min	Not required
PoND5	*Fat*I	1.0	55°C for 60 min	80°C for 20 min

3.7.3 Authentication of PCR Products of frankfurters by RFLP Analysis

To authenticate the four PCR products of beef and buffalo (Cocytb, CoND5, Bucytb, and BuND5) by RFLP analysis, beef and buffalo frankfurters were adulterated by spiking of 10% of buffalo and beef, respectively, and were heat-treated by boiling at 98 °C for 90 min and autoclaving at 121 °C under 15 psi pressure for 2.5 h. Porcine frankfurters were also boiled at 98°C for 90 min and autoclaved at 121°C under 15 psi pressure for 2.5 h, and RFLP analysis was performed in a separate assay.

3.8 Real-time PCR Assay

3.8.1 Design of Primers and Probes

The oligonucleotide primers designed for the conventional PCR targeting mitochondrial ND5 gene of cow (*Bos taurus*) and cytb gene of buffalo (*Bubalus bubalis*) and pig (*Sus scrofa*), respectively were used in the real-time PCR assay and the respective probe design also described in section 3.3.7 and they were listed in Table 3.12. Beef probe was labeled with HEX at the 5' end and ZEN/IOWA BLACK FQ at the 3' end; buffalo was labeled with TAMRA at the 5' end and TAO-IOWA BLACK RQ at the 3' end and pork probe was labeled with ROX at the 5' end and TAO-IOWA BLACK RQ at the 3' end (Table 3.12). Eukaryotic 18S rRNA specific primers and TaqMan probe (Table 3.12) were used as endogenous control (IAC) for the normalization and specificity test of the developed tetraplex qPCR assay (Ali et al., 2012b). The IAC probe was labeled with FAM at the 5′ end and ZEN/IOWA BLACK FQ at the 3′ end. The designed primers and probes were supplied by Integrated DNA Technologies (IDT), Singapore.

Table 3.12: Sequences and concentration of primer and probes used in this study.

Species	Target gene	Sequence (5′ - 3′)	Amplicon size (bp)	Final concentration (nM)	Reference
Cow	ND5	Forward: GGTTTCATTTTAGCAATAGCATGG Reverse: GTCCAATCAAGGGTATGTTTGAG Probe: Hex-ACAAATCTCAATACCTGAGACCTCCAACA GA-ZEN/IOWA BLACK FQ	106	500 500 250	This study
Buffalo	Cytb	Forward: GGGTTCTAGCCCTAGTTCTCTCT Reverse: ATGGCCGGAACATCATACTT Probe: TAMRA–AATCCTCATTCTCATGCCCCTGCTACA-TAO-IOWA BLACK RQ	90	300 300 200	This study
Pig	Cytb	Forward: TATCCCTTATATCGGAACAGACCTC Reverse: GCAGGAATAGGAGATGTACGG Probe: ROX-CCTGCCATTCATCATTACCGCCC- TAO-IOWA BLACK RQ	146	300 300 200	This study
Eukariotic (IAC)	18S rRNA	Forward: GGTAGT GACGAAAAATAACAATACAGGAC Reversed: ATACGCTATTGGAGCTGGAATTAC C Probe: FAM-AAGTGGACTCATTCCAATTACAGGGCCT- ZEN/IOWA BLACK FQ	141	200 200 100	Ali et al., 2012b

3.8.2 Multiplex (Tetraplex) Real-time PCR Conditions

Tetraplex real-time PCR assay of beef, buffalo, pork, and IAC were carried out in a Quant Studio 12K flex real-time PCR system (Applied Biosystems, Foster City, CA) in a 20 µL reaction volume consisting of 1× GoTaq Probe qPCR Master Mix (Promega, Madison), 30 ng of the total DNA template for each target species, and required quantity of nuclease free water. The concentration of primers and probes were listed in Table 3.12. The amplification was performed using initial denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 20 s, and annealing and extension at 60 °C for 60 s.

3.8.3 Specificity Test of mqPCR Assay

To analyze the specificity, the tetraplex PCR assay was carried out against the template DNA of 20 non-target species (goat, lamb, dog, cat, rabbit, monkey, donkey, chicken, duck, pigeon, quail, rat, salmon, tuna, cod, tilapia, rohu, pangas, frog and turtle) and 5 plant species (wheat, onion, garlic, ginger and pepper) which are commonly used in food.

3.8.4 Limit of Detection (LOD)

To determine the limit of detection (LOD) of the developed tetraplex qPCR assay, total DNA of the target species (cow, buffalo, and pig) were diluted using 10-fold serial dilutions (Cheng et al., 2014). Initially, a mixture with equal amount (1:1:1) of genomic template DNA extracted from the three target species (cow, buffalo, and pig) were made at 30 ng/µL. Then, it was serially diluted to 3, 0.3, 0.03, and 0.003 ng/µL of total DNA for three species with nuclease free water. Therefore, 3 µL of the each diluted DNA solution was added to 20 µL of multiplex reaction mixture so that each target species were 30, 3, 0.3, 0.03, and 0.003 ng of DNA in the reaction mixture and each diluted template was assayed in 6 replicates.

3.8.5 Generation of Standard Curves and Quantification of Target DNA and PCR Efficiency

To construct the standard curves of cow, buffalo, pig, and IAC from tetraplex qPCR system, DNA was extracted from the ternary admixture (1:1:1) of beef, buffalo and pork to form mixed DNA template in 1:1:1. After adjusting the concentration to 30 ng/µL (100%) the extracted DNA was 10-fold serially diluted to 3, 0.3, 0.03 and 0.003 ng/µL of total DNA with nuclease free water; this resulted in mixtures containing 100% to 0.001% of DNA for each species. Therefore, 3 µL of the each diluted DNA solution was added to 20 µL of multiplex reaction mixture as template and three closely spaced Ct values of the replicates out of six replicates (Cheng et al., 2014; Iwobi et al., 2015) were plotted against the logarithmic concentration of DNA for each target (Ali et al., 2012b). The efficiency of the assay was calculated from the slope of the standard curve according to the following equation (Druml, Mayer, Cichna-Markl, & Hochegger, 2015):

$$E\ (\%) = [10^{(-1/slope)} - 1] \times 100.$$

Acceptance range of PCR efficiency between 90 and 110%, corresponding to a slope of regression between -3.1 and -3.6, and R^2 value of ≥ 0.98 (Iwobi et al., 2015).

Quantity of beef, buffalo or pork in an unknown specimen was then determined by extrapolating the Ct value of the unknown sample in the standard curve for reference samples (López-Calleja, de la Cruz, González, García, & Martín, 2016). A semilogarithmic correlation was found between the variables, Ct value and concentration (Rojas et al., 2010):

Ct = m log [ ] + C

Where, m is the slope and c is the intercept.

3.8.6 Multiplex Real-time PCR Sensitivity and Validity

To evaluate the sensitivity and suitability of the tetraplex qPCR assay for food product analysis, two different model meat products (frankfurters and meatballs of beef, buffalo, and pork) were prepared in the laboratory (section 3.6.4) in three different days to check inter day reproducibility. Beef products were deliberately adulterated with 10, 1, and 0.1% (w/w) of buffalo and pork, buffalo products were adulterated with 10, 1, and 0.1% (w/w) of beef and pork, and pork products were adulterated with 10, 1, and 0.1% (w/w) of beef and buffalo (Table 3.8). The DNA was extracted from the meat products, and the concentration was adjusted to 30 ng/ μL using nuclease free deionized water and each template was assayed in 6 replicates.

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CHAPTER 4: RESULTS

4.1 Quality and Quantity of Extracted DNA

Total genomic DNA was extracted from raw meat, fish muscle tissues, admixtures and meat products (burger, meatball and frankfurter) on three different days. The concentration of the extracted DNA was determined based on the absorbance reading at 260 nm and its purity was evaluated based on the ratio of absorbance at 260 nm and 280 nm (Nejad, Tafvizi, Ebrahimi, & Hosseni, 2014). The absorbance at 260 nm indicates the absorbance maxima of Nucleic acids and that at 280 nm reflects the absorbance maxima of proteins. Finally, the A_{260}/A_{280} ratio provides the DNA purity indication with respect to the protein contamination (Oliveira, Paim, Reiter, Rieger, & D'azevedo, 2014). The A_{260}/A_{280} ratio in this study was found to be between 1.7 and 2.0 for all extracted DNA which ensured a high quality of DNA was obtained from all samples (Nejad et al., 2014). The amount of DNA extracted from animal and fish muscle tissue (20 mg) was 74−269 ng/µL, from plant species (100 mg) was 46−134 ng/µL, from meat products (200 mg) was 33−147 ng/µL and from heat treated samples was 32-125 ng/µL (Table 4.1).

Table 4.1: Concentration and purity of the extracted DNA.

Sample	**Average Concentration (ng/µl)**	**Purity (A_{260}/A_{280})**
Animal tissue (raw)	123−269	1.88-2.0
Animal tissue (Boiled)	71-125	1.80-1.96
Animal tissue (Microwaved)	32-54	1.78-1.85
Animal tissue (Autoclaved)	53-84	1.79-1.92
Fish tissue (raw)	74-161	1.84-2.0
Plant species (raw)	46−134	1.81-1.94
Meat products (raw)	84-147	1.78-1.91
Meat products (Boiled)	49-91	1.76-1.90
Meat products (Autoclaved)	33-62	1.70-1.92

4.2 Development of Biomarker

In this study, six pairs of primers (two pairs of each species) were designed targeting cytb and ND5 genes of cow, buffalo, and pig species to develop a double gene targeted mPCR assay with short length of amplicons (Table 3.1). To develop multiplex PCR assay with successful PCR products the designed primer pairs must have the particular criteria including short length amplicon (≥ 150), fully matching with target DNA and not matching with non-target DNA, with 40-60% GC content and Tm between 55-60^0 C (Rashid, 2015a).

4.2.1 In-silico Analysis of Biomarkers using Bioinformatics Tools

The designed primer sequences were aligned in silico against the similar regions of target and 28 non-target species including 16 terrestrial animal, 8 fish, and 4 plant species, as cited in section 3.3.7. Complete sequence matching was found only with cow, buffalo, and pig species, and 3−18 nucleotide (12.5−80%) mismatches were found with other species (Table 4.2- 4.7). The pairwise distance was also computed using the neighbour-joining method (section 3.3.8); the lowest distance (0.144) was observed between the cow and goat species, and the highest (1.993) was found between the cow and wheat species (Table 4.8-13). These indicated adequate genetic distances among the studied species, eliminating the probability of any cross-target detection (Taboada et al., 2014). Moreover, the analysis of phylogenetic trees (Figure 4.1 (a)-(f)) and 3D plots (Figure 4.2 (a)-(f)) demonstrated similar findings, supporting the results of other in silico tests.

Table 4.2: The mismatch comparison of the beef cytb-specific 120 bp site against other 28 non-target species.

Species	Forward	Middle sequence	Probe	Middle sequence	Reversed	Mismatch		
Cocytb	CGGCACAAATTTAGTCGAAT	GAATCTGAGGCGGATTCTCAGTAGACAAAGCAAC	CCTTACCCGATTCTTCGCTTTCCATTTT	ATCCTTCCATTTATCATC	CATAGCAATTGCCATAGTCCA	FP	RP	Pb
Cow	....	....	....	....	....	0	0	0
Buffalo	T..T....G.C.G..T..G.	....T.....G.....	C.....A..T..C..C	.....C.....C..T..	GC....C....A.....	7	4	5
Goat	T.....CC.....	.....G..... ....C..	T..C.....C.....C.	.....C.....C.....	..C...CC.C.....	3	4	4
Sheep	T.....CC.....	.....A..... ....T..	...C.....T.....C..T..C....	TT.C.....C.....	.GC...CC.C.....T..	3	6	5
Deer	T.....CC.....	.....G..C..T.....T.....	...A.....T.....C....	TT.....C.....	.GC....C.C..T.....A..	3	6	3
Donkey	...T..T.CGC.C.....	.....T..... ....C..	.....T..T..C.....C....	T..A..C.....	..C...CC.G.TA..C.....	6	7	4
Horse	...T..T.CCC.C.....G.	.....T..... ....C..	.....T..T.....C..C	.....A..C..C.....	..C...CC.G.TAG.C..A..	7	9	4
Pig	...A...G.CC.C..A.....	.....G..C..T..C..C.....	...C..A.....C.....C...	.....G.....C.....	T.CC..CC.C..AGCC..A..	6	11	4
Dog	...A..TG.C.....A.....	.G.....C.....G.....	...A..A.....T..A.....C	.....C..T..C.....	.GC...TC.A..A.....A..	5	7	5
Cat	...G..TG.AC....A.....	.....G..G..... .....C..	...A..A.....T.GC.....C..C	..T.....C..T...	TC....CT.A..AGG...A..	6	9	7
Rabbit	...A....CC.....T.....	.....A.....T.....T..T.....C..	T.....T..C..C	...T.G.....	TGC.A.TT.A.T.T..A.T..	4	11	4
Monkey	...A..T..CC.T...C.....	.....A....A.G.CA.T....GCC.C.....	....C..A.....A.CC.G..C.....	.....A..C..C.....	....C..CC.C..AG.C..A..	6	8	7
Chicken	T..ACAC.CCC....A..G...	GC.....G.....T.....C.....CC.....	.....A..C..C	C.....C..C...GCA...	GC..GT...A.T..CA.....	10	8	3
Duck	...ACAG.CCC.G..A.....	.GC.....A.....G..T..CC.....	....A.....CA.T..C..C	C.A..A..C...T.A...	GC..G...CA..C.....	8	6	6
Pigeon	T...CA..CCC.C..T.....	.GC.....T..C.....T..CC.T..AT.A..A.....	.A.CC.T..C..C	C.....C..C.....A...	GC..GCC.CA.T..CA.....	8	10	10
Quial	....CA..CCC....A.....	.GC.....A..C..T.....T.....TC.T.....	.....CC.....C..C	C.....C.....CT.A...	GC..G...CA.T..CA.....	6	8	5
Rat	T..G..T.CCC.....	.....A..C.....	....A..A..C.....A.....C..C	.....C.....C..T...	.GCC...CC.....A..T..A..	6	8	6
Salmon	A..AGGCGCCC....AC...	.....G.....T..T.....C..T..T..A..A.....T.....C..T..C..C	C.AT.C..C..CG....	TGC...TGC.A.AG.CC.....	11	11	8
Tuna	T..A..T.CCC.C..T.....	.....G..C..T.....T..T..	.....A.....C..C	C.AT.C.....CG.....	.GC...C..GA.A..TC.T..	8	9	3
Cord	A..TGATGCC.....TC....G	.....A..T.....T..T..T....A..T..G..T..T..A.....CT.AT.C..C...G.TG.	TGCT..TT..A.A...C.....	10	9	7
Tilapia	T.....ATTCC.....C.....	.....G.....C.....T..C....A..T..C..T.....C.....CC	..T.C..C..C.....TGC...TGCAA.A.....	7	9	6
Rohu	A..AGAC.TA.....C.....	.....T.....T..C.....T..C..A..A..A..T.....A.....C..C	C.A..A.....	TGCC..CGCAA..C.TA.T..	8	13	7
Pangas	A..AGAC.CCC....AC.....	.....T.....G..C.....T.....T..C.....A..A..A.....A.....C..C	C.A..C.....CG.AG.	T..C...GC.A.AC..C.A..	10	9	6
Frog	....T.T..CC.T...C....	.G.....A..C.....C..T.....C.....T..TA.A..T..C..C	..T..C.....T..T.CC..TG..AGTC..A.T..	6	11	8
Turtle	.....AC.CC.....AC.....	.....T..C.....C..T.....A.....A.CC.....C..C	C.A..A.....GCT.CT..T.GGT.G..T.....A..	6	8	6
Wheat	A..GC.TG.....TCTCT..C	..GA...TT.TA....GTCAG.T.TT.-TTT.TT..AGTATCTGGAACACGAAATAGA.AG	.GTGGAT..AAA.AA.ATGG.A.T.....G..TCATAC	10	12	25
Onion	TATA..T...CC..CTCT..	.....TTG.AT.GAA..AG.T..G.-CTT.TTTGA.....	.ATGAA.TG.A.GA	.CAAA.TA..AA.AATAA...TTTC.....C.TCAGGT	10	12	14
Ginger	T.CAGATG..A..TAACT.G	.....A.ATAT.T.C...CTC.TGTC..CAT.TTT.ATTGTTTAGG..G.GGA..ACAC...	CTTG.TTT..AG.ACA	AG.....T.CA.GTT.T.CTAT	14	13	20
Chilli	T.CGGATG..A..TAACT.G	.....A.ATAC.T.C.C.CCC.CGTC..TAT.TTT.ATTGTTTAGG..G.GAA.TACGC...	CTTG.TTT..AG.ACA	AG.....T.C..GGT.T.CTAT	14	12	21

Note: FP: forward primer, RP: reversed primer and Pb: probe

Table 4.3: The mismatch comparison of the beef ND5-specific 106 bp site against other 28 non-target species.

Species	Forward		Probe	Midle sequence	Reversed	Mismatch		
CoND5	GGTTTCATTTAGCAATAGCATGG	TTCCTA	ACAAATCTCAATACCTGAGACCTCCAACAGA	TCTTCATACTAAACCCAAGCGA	CTCAAACATACCCTTGATTGGAC	FP	RP	Pb
Cow	.....	.....	.....	.....	.....	0	0	0
Buffalo	..A..T..CC.....A	.....	G.....T..C.....A	.....GA.A	...T...G.....C.A..A....	5	5	4
Goat	..A.....CC.G.....A	.....	GTC..C..T..C..T..G.....T.....A	.....T.....A...GA.A	.....TC.....C.A..A..CT	5	7	10
Sheep	..A..T...C....C.....A	..T....	.TT..C.....C.....T.....A	.....T.....T....A	.....C.....C.A..A....	5	4	6
Deer	.....T..CC.....A	C	.....T..CG.....T.....T..TGC...G.CA....A.A	...C.....C.A..G..C.	4	5	4	
Donkey	.....CA....C.....C..A	.....	TTC..CAC...C..A.....A.....A	.....TG....T.A.CT.A.C...C.C..AC.CT.A..T.	5	10	10	
Horse	..C.....CA.G..C.....C..A	.....	TTC..CAC...C..A.....A	.....CG.....C.A.CTTA.C...C.C..GC.CC.A..C.	7	11	9	
Pig	..A..TG.CC..T.C.....A	.....	...CC.CTCA..CG.A.....T..T.....A	.....T.....AATGAATG	.C.....A..A..C..C.	8	5	12
Dog	..C.....A..A.C.....A	..T...	CT...CT.A..C..A.....T.....A	.....TACG.CAAACGATA	.T.TT..TC.G..AC.AC.....C.	5	11	9
Cat	..C.....CA....C.....A	..T..C	..C..CTCA..CG.A..G...T.....A	.....T..CACCC.A.ACGAGA	..CT...T..T..A..AC.A..G.	5	9	11
Rabbit	..C..T...A....CT....C..A	..TGCT	.TT..C.....C..A.....A.....A	.....T..TT..G..AAT.A.ATTA	.C.T.C.C..AC.....A...T	7	10	7
Monkey	...C.T..CC.G..C...A....A	..T..C	CT.C..TA...CT.A.....A	..AC.AGCC.....T.C.A.TC	AAGCTC.C.T..A...G.AA.C.	8	13	9
Chicken	..CC....CC..AGC.....A	C.AGCC	T.TTCC..A..C.....AA.T.....A	.-----.CC.ACCCAAACCAAAC	AC.C.C.C.C...C.CC.....C.	8	10	11
Duck	..CC.A..CC.GAGC.....A	C.AGC.	T...CCT....C.....AA.T.....AG	C----.G...ACCC..ACCAAAC	.C.C.T.C.C...C.CC.A....	10	9	10
Pigeon	...C.A..CC..AGT.....C..A	C.TGCT	T.C.C.ACA..C.....AA.....A	C---A.CCTCT.C.A.CCAAAC	AC.T.TTC.C..TC.AC.A..C.	9	13	10
Quial	..C..G..CC..AGCC.....A	C.AGCC	T.T.CAT.A..C..A....AA.....A	C---T..T.ACCC..ACCAAAC	AC...CTC.T...C.CC.....C.	9	10	11
Rat	.....CC....T...A.C..A	...TGC	CT...CA.A..CT.....A.....A	..T...T..ACC..TA.C.A.A	TCT.G----.C..TC.C.CA....	6	10	9
Salmon	..AC....CC..AGT.....C..	...GC.	.....C..A..CT.A....AA.T.....A	..A.C.TCTTC...AGG.CTT	...CT..CAC.C..TC.TT.G..C.	8	12	9
Tuna	..AC.....C.T..C.....AA	.AGC..	.T..C..A..CT.A....AA.A.....A	..A..A.CCGCT..AAACTT	...TCT..CTC.C...C.CC.A....	6	11	10
Cord	.....A..CC...GT..G....AC	.AGCT.	.T..CG.T..C.G...G..TA.T.....A	..A..TT..AGC..GGATTTG	...AT..CTC.C..TC.AC.C..TT	7	13	11
Tilapia	..CC.A.....T..T.....T..AA	..GC..	.TTCC.....CT.T.....AA.A.....A	..A.CTT..TCT..AGATTTT	..T.T..CTTAC...C.TG....C.	7	11	11
Rohu	..AC.A...A....C.....C..AC	.TGC..	T...C.....CT.A.....AA.....A	.T.C.T..TC...AAACTTT	...ATG.C...C...C.A..A....	7	8	8
Pangas	..CC.A...C.TA.T..T..C..AA	.TGC..	.....C.....CT.A.....AA.T.C...A	.....AC..GCC..AGA.TT	....AT..CAC.T...C.A..G..CA	10	11	9
Frog	..C...C.AC..T.CT.TTGC..A	..TT.T	.A....GC.C.A..A.T.....T..GCC..T.	.TC-TC.CGCT.GAAACT.GCC	ACTG.....C.TC....GT	12	9	12
Turtle	..AC.A...C.CAGC...T....A	C.AGC.	.T....CACA..C.....A..A.....A	..A..T.CCAAT.CTAACCCAAC	.C..CTAC.C..AC.TC....C.	10	11	9
Wheat	.AAC..TC.....TGC..CTG.AA	..TTG.	.A.TGAACACGC.AA.ACCCATCAA.TGTA	.GTA..CG.AAC.TATA..AG.C	AGTTT..TCCTG.AG.A.AG.AGG	13	17	25
Onion	.AAC.AT.....GC..CTG.AA	..TTG.	.A.TGAA.ACG..AA..TCCATCAA.GGTA	.GTA.TCG.AAC.TATA..AT.C	AGTG..TCCTG.TG.A.A..A.A	12	15	21
Ginger	.AGC.AT.A..G.T.C..CTG.AA	..TT..	CA.TT.A.ACGC.AA..CCCATCAA..GTA	.GTACGCG.AAC.TATA..AT.C	AGTT..TCCTG.TG...AACA.G	14	16	22
Chilli	..GG.TT.G.GTTTCCC.C...AA	..TTG.	.A.TGAACGTT..AA..CCCTTCAA..GTA	.GTA..CG.AAC.TATA..AT.C	GGTT..TCCCG..G..GCCCA.G	15	16	22

Table 4.4: The mismatch comparison of the buffalo cytb-specific 90 bp site against other 28 non-target species.

Species	Forward Primer		Probe	Middle sequence	Reversed Primer	Mismatch		
Bucytb	GGGTTCTAGCCCTAGTTCTCTCT	ATCCT	AATCCTCATTCTCATGCCCCTGCTACA	TACATCCAAACAACG	AAGTATGATGTTCCGGCCAT	FP	RP	Pb
Buffalo	....	.....	....	....	....	0	0	0
Cow	.A..A..........CCT.....	.....	...T..TGC...A..C.....A.....	C..C...........	...C..A..A.....A...C	5	5	7
Goat	.A..C...........C.....A	.....	....T.AG.A..TG.A...T.C..C..	C.....T........	...C..A..A.....C...A	4	5	10
Sheep	.A..C..C......A.C.....A	.....	.G....AG.AA.T..A.....C..C..	......A..G.....	G..C..A..A.....A...A	6	6	9
Deer	.A..CT..........CTCA...	.....	....T.G...........TT.T..T..	C..G...........	C..C.....A.....A....	7	4	6
Donkey	.T..AT.......TA.C..T..C	...T.	......AGCA.....C..TACC.....	C.TG..A........	...C..A........A..CC	8	5	9
Horse	.C..AT........A.C.....C	.....	G.....AGCA.....C...ACC..C..	C.T...A........	...C..A..A........TC	6	5	10
Pig	.A..GT.G........AGC...C	.....	......A...T.A.......A.A....	C..............	...C..A..A..T..A...C	8	6	5
Dog	.T..A..C........AT....C	.....	....T.GGCAT....T..A..C..C..	C.....T..G.....	C..C..A..A........CC	6	6	10
Cat	.A..C...........A.....C	.....	.G.A..AGCAA....T..AA.C..C..	C..C...........	.G.A..A.....T..A...C	4	6	12
Rabbit	.A..C.....T.....C..A..C	.....	TG.T..AGCCT....C..AT.C..T..	..TG..T........	T..C.....A.....A..CA	6	6	13
Monkey	.C..A.....AT..T.C..A..A	...T.	C...T.AGCAGC...C...A.A..T..	C.A...........A	......A..A.....C...C	8	4	12
Chicken	.T..A..........CAGC...A	G....	C......T.C..A..C...T.C..C..	C.A...T........	..CA..A.CC.....A...C	7	7	8
Duck	.C..C.....A....CCGC...C	G....	......AT.C..GG.C...T.C..C..	C.A...A........	..CA..A.CA........GC	8	7	9
Pigeon	.A..A......T...CCGC...A	G.A..	...T..AT.C....CC.....A..C..	C.AG...........	C.CA..A..C.....C...C	8	7	8
Quial	.C..A..T.....T.CAGC...A	G.A..	T......C....A..C...T.C..C.C	C.A...T........	..CC..A.CA.....C...C	9	7	8
Rat	.A..CG........A.CT.A..A	.....	....T.AGCCT..C.A..AT.C..G..	...T..A........	C..CT.A.CA.....C...A	8	8	12
Salmon	....A..C.....TT.AT....G	.....	TG....T..AG.TG.T..TA.CT....	C..T...........	.G.AC.A.CC..T..A...C	7	9	12
Tuna	.A..A........CC.AGC...C	.....	CG.A..T..AG.AG.C...T.C.....	C..T..A.....G..	..C.C.A.CA.....A...G	8	7	11
Cord	.C..A..T..A..CC.AT....G	..T..	.G.......GG.TG.....T.T..C..	...G..A........	.G..T.A.CA.....C..TC	9	8	8
Tilapia	.A..C..C.....CT.AT....A	.....	TG....G..AG.TG.A...A.T..C..	...C..T........	.G.CC.A.CC.....C..TA	8	9	11
Rohu	.A..C..T..AT..C.AT....A	..T..	.G.AT.A..AG.AG.......C.....	C..C..A........	.G.AC.A.CA.....C...A	9	8	9
Pangas	.A..A........GC.AT.....	.....	.G.AT.A..AG.TG.C.....AT....	C..C........G.A	.G.AC.C.CC.....C..CC	6	9	11
Frog	.A..C........TC.C..A..A	..T..	GG.T...T.C..G..A.....CACT..	...C..T....TT..	CTCCC.T..A..T..C..CA	7	11	11
Turtle	.T.....C.....CT.AT....C	..T..	.GCAT.AC.C..A..A..AACC..C..	C.....A........	CTCA.CT..A..T..A..C.	7	10	14
Wheat	TCAAAA.T.TGAC..G.G.AC..	GA.GC	C..T.CGG.AA.AGGAT.G.CTT..GT	GGAG.TATT..GCG.	....GCT.GTG.GG.C.A..	15	10	18
Onion	A.....G.T.TA.G..CA.TGG.	GG...	......A.C..CT.A.GAT..A...A.	.T....A.GT...A.	..TC.AA.C.G.....TT..	11	8	10
Ginger	TCAAAA.T.TGACG.GCG.AC.C	GAAGC	T..T.CTGGAA.AGGAT.G.CTT.GGT	AGAG.TATT..GTG.	....GCT.GTG.GG.C.A..	18	10	20
Chilli	T.AAAA...TAAC..G.G..C..	GA.GC	T..T.CTG.AA.AGGAT.A.CTT.GGT	CGA..TATTG.GCG.	...CGCT.GTG.GG.A.A..	12	11	19

Table 4.5: The mismatch comparison of the buffalo ND5-specific 138 bp site against other 28 non-target species.

Species	Forward Primer	Middle sequence	Middle sequence	Reversed Primer	Mismatch	
BuND5	TCGCCTAGCTTCTTACACAAAC	TTAACGATAAGCCAAAAACTAGCATCCTCTCTCCTAGACCTAATCTGACTAGAAAACATTTTACCAAAAACCACCTCACTTATCCAGATGAAAGC		ATCCATCACAGTCACAAACCA	FP	RP
Buffalo	.....	.....		.....	0	0
Cow	.....C.A....T...TC..T.A.....TC.....C..T.....GC...CC.....T.....CGC...A..A.....T.C.CTG.....				4	5
Goat	C...T..ATGC.C....T....C....A.....TC.....C.....T.....GC...CC.....TT.....AGC...A..A...AT.G.T.C....A.....G...				8	5
Sheep	C.....GA.CC.C..T.T....C....A.....TC.....T.....CT..CC.....TT.....AGC...A..A...AT.....C....A.....G...				8	3
Deer	C.....G..CC.....T...TC....A.....TC.....C.....T.....C.....T.....A..T.....A.CA...AT...A....TG..T.....				6	4
Donkey	...A..CC.AC.ACT.G.....C..T.A.....TC.....ACT...A.....TC.....G.....CC.....T...T...C.AAT.T..A..A..GA.C..A...CT.A.TT.C.C...				9	9
Horse	C..A..CC.AC.GCT.G.T...C..T.A.....TC.....ACT...A.....TC.....CC.G.....T.T.T...C.AGT....A..A...A.C..G...CT.A.TT.C.C...				11	9
Pig	C.....C.AA.A...CAC...C..T.T.....TCC.....A..AT.A.....T....T.....CT...C.....A.....TT.C.....A..A...AT...A....T...AT....T..				8	5
Dog	C.....CA.AC..A.A....GT.....C..T.....G...TC.....TATA..T..G...TCC.....CC....C...T.A.T....TA.T....A..A...T.T..T.C.CTTA.TT....T..				9	10
Cat	C.....CC.A..AA.A.TG.G.C....T..G.....GTCC.....GATA..AT.....A....T.....TG.A.....C...T...T...CT.AT....A..A...ATG..A.CT..T..AT.T..T..				10	9
Rabbit	...TA.TCTAC.C..TCT....C.TT.AGCT.....CA....CA.A.TA.....TA.G.C....ACT.....AGCGA.C..G....AT.T....GAC..T..A..TTTC.....A.C.T.C..AT.CTCA..				11	10
Monkey	C...AC.ATCC.C..TCA....C..CTC.....TT...TCCT.CTC..A.....TC..CA.....ATC.A....T.....A.T.....AAGCA..C..C.CC..T.....A..TA.A..C.C...				11	7
Chicken	C..AA.TAGCC.C.CA.TCCT.C..CAT.CCG.A.....A.T.....CACT.AA.C...A..GCA..GTACA....A..AGGC..CG..GG.CTTG.CAACC.T..TC.C.CCAT.A.....T..AC....CT...				15	6
Duck	C..AT.CTGC..CA.A..CCTGC..GAA.AGG.G.....CA.C..CCTACAC..AA.C.....CTC...G..CA....A..AGGG...G.GGG.CT.G.CGAGC.G...G.AGCC...AG..C.G..AC.CTGGCA..				14	12
Pigeon	C..A..TAGC..AACAGGCCT.C..CTCTCCG.A.....A.C..C...CAC..AA.T...T..TC....TACA....A..AGGC..TG..GGACT.G..GACC....AC.C.T....G...C.T..A...ACCTT..				15	9
Quial	...AA.CAGCC.CACA.TCTCAC.CTAC.CCG.A.....A.C..C..ACT...AA.C...A...CA..GTACA....A..AGGC...G..GGACT.G.TAACC.A..CC.C.TCAT.A...C.CT.ACT....C...				16	8
Rat	C..AA.TAT.C..C.A.A..CTC...ATTCT...T.C...T..T.C.TAAAC..A.....G.CAA.C.....T.A.....A.C.C...A.CCC..CT...T.AA.T.A.AT.C.....				12	8
Salmon	C..AT.G..CC.AA.ACT.....TC..G.A....CCA.T..TAG.CAAA.GG....T.A..CA...T.T.....AG.CGGC.....GGAGTTA.TT.AAC..ACCTACCC.AT.GT..CA...AGT.AT.T...				11	10
Tuna	C..TT.CA.AC..A.ACTC...C..GTAT..G.....GCCA.T..CAGTCAAA.AG.T....A..C.....ATCAGGC..T...G.TTTAG.CACATCAA.CC.TCCTCT.ATT.CA...AG..ATGC...				12	11
Cord	C....A.AT.C..C.ACTC.G.C.CCTTC..G.A.....A.T..TAG.CAGA.AG....T.AG.CA.....G.C.GGT..C...G.T.TTG.TAA.GCTACC.CCCCTCT.G..TCA...AG..AC.TA..				11	10
Tilapia	.....T...C.CA.ACTT...C..GTTT..G.A....CCA.T..C...CAAACAG.C....A..CT..GT.....AG.CGGT.....G.A..TG.CACCC..A.CC.CCCCCT.ATT.CA..CAA..AT.TT..				8	12
Rohu	C..A..CCTAC.AA.ACT....C.C..CC.CG.A...TC.ACT..CA..CAA.----.....AA..CA.....T.G..CA...GGGA.....GGGCTAGG...AGCA..A.CA.CCAT.G.A..T...AA.GAC.T.AC				12	11
Pangas	C..A..CATCC.AA.ACTC...C.C..AT..G.AA..C.-----.CA.TAAA.----.T....G.CAA...A.G....TA...GG...C...GGACTTCAC.CCC....CCACTCCTTG...T.ATTT.A..AC.TTG.				13	12
Frog	C..AACC.TCGTCACGGTC.CTC.CGAC.C.GT.A..C...A.CCT.GA.CAAG.....TGC.T...A.A..A.TT..TAGGCC.TTGTCC.T.TAC.TTCGGCT..A.ACCC.TTGAG...AGT.CGTGTTTC...				18	14
Turtle	.....CTCC.AATA..C...C...AAT.C.....CA.T...ATTAAC..AA.C...A.GTC....TAT.....T..CGGC.....GGTCTAATTAAAACCA..A..C.CCAT.AT.....TCTC.T..TCA..				9	10
Wheat	AGTAAGT.GG..AAT..AGT.TCC..ATTCT.AAG.....TC.TT..TGA.GG...A.....AT.GA.ATTG.T.G.T.GAAC.T...TTT.TTTGT.G.A.AGA.AGTTGA.CT.AGAAT..AG.TA.ACTT..GAG				16	15
Onion	AGTAAGC.GA..AAT..AGTGTCCG.ACTCT.AAG.....TC.TT..TGA.AG...A.....AT.GA.ATTG.T.G.TA.AA..T..GTTT.TTTGT.G.A.AGAAAGAT.A.C..A.AAT..AG.TA.AGTT.G.AT				17	14
Ginger	A.TAAGT.GA..GATT.A.T.TCCG.ACTCT.AGG.....TC.TT..TGATGG...A.....AT.GA.ATTG.T.G.TGGAAC.T...TTT.TTT.T.G.A.AGATA.AT..GCT.A.AAT..AG.TA.ACTT..GA.				15	13
Chilli	AGTAAGT.GG..GAT..AGT.TCCG.A.TCT.AAG.....TC.TT..TAATGA...A.....AT.CA.ATTG.....AGAAC.G.T.TTT.TTTG..G.A.AGA.AG.TA..TT.A.AAA..G..TA.GCTT...A.				16	11

Table 4.6: The mismatch comparison of the pork cytb-specific 146 bp site against other 28 non-target species.

Species	Forward	Middle sequence	Probe		Reversed	Mismatch		
Pocytb	tatccctt atatcggaacagacctc	gtagaatgaatctgaggggg ctttccgtcgacaaagcaaccctcacacgattcttcgccttccacttt atcctgccattcatcattaccgccctcgcag	ccgtacatctcctattcctgc			FP	RP	Pb
Pig	.....	.....	.....		.....	0	0	0
Buffalo	a.....a..c..t..t...agt..g	..t..g.....t.....a..c..a..a.....c.....a..t.....c.....t..cg.a..a..t...a	ta..c..c..at....t..c.			9	8	6
Cow	a.....a..c.....c...a.tt.a	..c.....c..a..c..a..a.....t..c.....t.....t.....t.....c.ta..aa.t..ca	ta..c..c..a.....c.			8	6	7
Goat	a.....a.....t..c...a....a	..c.....a..c..a..a.....c..t.....c.....c.....c..a.....ca	ta..c..c..g..t.....c.			6	7	3
Sheep	a..t..a.....t..c...a....a	..c.....a..a..c..a..a.....t.....c.....t.....t.....tt.c.....cg.a.....ca	ta..t..c..a..c.....c.			7	7	6
Deer	a..t..a..c..t..c...a....a	..c.....a..a..t.....a..c.....t.....t.....tt.t.....cg.a..a.....ta	ta.....t.a..c.....t.			8	6	7
Donkey	a.....c..c.....t..tacg...	..c.....t..a..c..a..a.....c.....t..c.....t..t.....t..a..c..t.....c..a.....g.t.at...c.....a.....c.				8	4	6
Horse	a..t..c..c.....t..tac.....	..c..g.....t..a..c..a..a.....c.....t..c.....t..t..t.....c.....a..c.....c..a.....g.t..t.....t.a.....t..t.				8	5	4
Dog	c.....t...t.a	.....g.....c.....c..a..g.....a.....t..a.....t..c.....c..t.....cg.a..t..a...a	ta.....c.....t..a.			4	5	4
Cat	a..t..a..c.....g..t..a..a	.....g..c..a..a.....c.....a.....t.g.....c..t..t.....t..ct.a...t.a....ga.....c...t.....t.				8	5	7
Rabbit	a.....a.....ac.t.a	..t.....a..a.....a..t..t.....c..t..t..c.....t..t.....c...t.....t.....g.aa.tt.a.tct	taa.t..c.....c..t..a.			6	8	7
Monkey	a.....g..c.....ta....t	..cc.....a..a.acg..a.t....gcc.c.....a..c.g.....a..c.....c.t.....t.....ct.a.....a.				6	5	4
Chicken	a..t..c..c..t...cacac...a	.....g...gc.....a.....a.....cc.....t..c.....t..a.....cc....c..c..tgca..cg.a.gta.ta.tat.a.c..c...acc.....a.				11	8	12
Duck	cc....a..c.....cagac...g	.....gc.....a..a..c..a..g..t..cc.....a..c.....a.t.....cc.a..a..c..tt.a..cg.a.gaa..a.cc	ta..c..ct.aacc.....a.			10	10	12
Pigeon	.g....c..c..t..cca.ac.....t	.....gc.....c..a.....a..t..cc.t..at.a.....a..c.t.....cc....c..c..t..a..cg.a.g....a.ta	t.a.c..c...acc.....			9	7	8
Quial	.g.a..c..c.....cca.ac...a	.....gc.....a.....a..t....tc.t.....a..c.....c.....cc....c.....t.a..cg.a.gaa..a.ta	t.a.c..c...ac.....a.			10	7	9
Rat	.....c..t..g..tac...a	..c.....a.....c..a..a.....a.....c.....a.....c.....c.....t..cg.....t...att.....t..t.....c.				7	5	4
Salmon	.g....g..cg.a...ggc.c...a	...c.....c..g..c..t..t.....c..t..t..a.....t.....t.....cc.at.c..c...g.....g.a..tgc	ta...t.c.c..c..t..g.....t.			10	7	10
Tuna	ag....a...g.t.....tac.....	.....t.....a..a.....t..t.....t..c.....a.....cc.at.c.....g....cg.a...a.ga..attc.t..c..t..t.....t.				8	8	8
Cord	.g.t.....g.a..tgat.c.t.a	..tc....g.....a..t..c..a..a..t..t..t.....a..t..g..t..t..a.....t..ct.at.c..c..tg.tg..g.t..tt.ta..atac.c..c..a..t..t..c.				11	9	12
Tilapia	a.....c..t..c.attc.t.a	..cc.....a..c.....a.....t..c.....a..t..c..t.....t..cc..t.c..c.....g.a..tgc	aa..ata..c..ct.aa.t..t..t.			10	10	8
Rohu	cg.a..a..c..a...gacatat.a	..cc.....t.....t.....c..a..a.....t..c..a..a.....t.....a.....cc.a..a.....t.....g.....gc	aa.cctta.t..c.....c.			14	6	6
Pangas	cg....c.....a...gacac...a	...c.....t.....c..t..a..t..c.....a..a.....a.....cc.a..c.....g.ag...t...agc	ta..ctac.....gc...g..t..a.			10	8	9
Frog	cgc...c..c.....ct.ta....t	..cc....g.....a.....c..a..a.....c..t.....c.....t..ta.a..t.....c..t..c.....t..t.....tg.tagtc	taa.t..c.....tt.a.			10	8	6
Turtle	c.c.....c.....c.acac.t.a	...c.....t.....c..a..a.....c..t.....a..c.....a..c.....cc.a..a.....tgct.c..tt.ggt.g..ta	ta.....ct.a..c.....a.			10	7	12
Wheat	a.cgt...gagattc.ggca.ttg	ag.tg..at.a..agtaaata.g....tc.ccatgt.....at...tttt...g..tagggga.t.ac.c	ttacttg..ttct.g...aagt.g.tacc	ggt.ttgctatgac..tt.a.		18	16	17
Onion	...a.t.cggt.tttt.tt.g.tg	.t.ca.c.at.a..c-ca.ct..a..gat.g..ttg.a...gat....gac-..a..t.aaa.ga.t.ga..gaacaa...----.a..aaaataa	atattt.tccttcaggtat.ctac.g			15	18	14
Ginger	a.cgt..cgagattc.ggc..ttg	ag.tg..at.a..agtaaata.g....tc.tcatgt.....at...tttt...g..taggggg.tcac.c	ttacttg..tt.t.g...aagtag.taca	ggt.ttgctatgac..tt.a.		18	16	17
Chilli	a.cgt..cgagattc..gca.ttg	.g.tg..at.a..agtaaatacg....cc.ccacgt....tat...tttt...g..taggggga.t.acgc	ttacttg..tt.t.g...aagtag.tact	gg..ttgctatgac..tt.a.		18	15	17

Table 4.7: The mismatch comparison of the pork ND5-specific 73 bp site against other 28 non-target species.

Species	Forward primer	Middle sequence	Reversed primer	Mismatch	
PoND5	GATTCCTAACCCACTCAAACG	CATGAGATCTTCAACAAATCTTTATACTAAA	CAATGAATGCCCAAACATACC	FP	RP
Pig	.....	.....	.....	0	0
Cow	.G.....AA.TCTC..TA	.C.....C..C.....G.....C.....	.CCAAGCGA.T.....	9	9
Buffalo	.....G.AA.TCTT...A	.C.....C..C.....C.....	.CCA..CAA.T.T...G....	8	9
Goat	.....GT.A..CTT...A	.T..G..C.....	ACCA..CAA.T....TC....	7	10
Sheep	....T....TTA..CTC...A	.C.....C.....	TCCAA.CGA.T.....C....	8	10
Deer	.....C..AA.TCTT....	.C.....CT.C.....G..T...GC...G.C	ACCAA.CAA.T.C.....	7	10
Donkey	.....TT.A..A.C...A	.....C..A.....C.....TG.	.CC.A.CCT.A.C...C.C..	6	10
Horse	.....TT.A..A.C...A	.....C..C.....C.....CG.	.CCCA.CCTTA.C...C.C..	6	12
Dog	....T...CTAA...T....A	.....C.....C..TACG.C	A..C..TAATTTT..TC.G..	7	12
Cat	....T..C...A.....	....G..CT.C.....CACCC.	AC.C..GAA..T...T..T..	3	9
Rabbit	....TGCT.TTA..CTC...A	.....A..C.....TT..G.	....A.CATTA.C.T.C.C..	11	10
Monkey	....T..CCTA..T.AC...T	.....C..C.....AC.....GCCCT	A..CTCCAA.T...C.C.T..	9	11
Chicken	..C.AGCCT.TTC.CT....A	.C.....AA.....CC.ACCC	A..CC..ACA..C.C.C.C..	12	10
Duck	..C.AGC.T.AAC..TC...A	.C.....AA.....GC....G...ACCC	.C.CC..AC...C.T.C.C..	11	9
Pigeon	..C.TGCTT..ACTA.....A	.C.....AA.C.....C.....CCTCT.C	..CCC..ACA..T.TTC.C..	11	11
Quial	..C.AGCCT.TACA.T....A	.....AA.C.....C.....T.ACCC	.C.CC..ACA....CTC.T..	12	10
Rat	.....TGCCTAA..AT....T	.C.....A...C.....T..CT..ACC..	T.CCA.CAAT.T.GT---C..	10	11
Salmon	.G...GC...AA..CT....T	.....AA.....A...TCTTC...	AGGACTTGA..T..CAC.C..	8	14
Tuna	..A.AGC...TA..CT....T	.....AA.A.....A..C.CCGCT..	A..CTTCGAT.T..CTC.C..	9	13
Cord	..C.AGCT..TA..GTT...AGC..G...A.....A...T..AGC..	GG..TTGGA.AT..CTC.C..	11	13
Tilapia	..A..GC...TTC.CTC...T	.T.....AA.A.....A...T..TCT..	AG..TTTGATTT..CTTAC..	10	15
Rohu	..C.TGC..TAA..CTC...T	.....AA.C.....T..CT..TC...	A..CTTTGA.ATG.C...C..	11	12
Pangas	..A.TGC...AA..CTC...T	.....AA...C.....CC..GCC..	AG.ATTCGA.AT..CAC.T..	10	14
Frog	....TT.T.AAA.TG.CC.AA	..T...CT.CGCC..T..TC.C-.CGCT.G	A..CTGGCCA.TG.C.....	12	11
Turtle	..C.AGC..TAA..A.....A	.C.....A..A.....A.CC.CCAAT.C	T..CCC.AC....CTAC.C..	9	11
Wheat	A...TTG....A.TGA.C.....AATACC.A.....-.TG.AAG...C.G...	..TAT..AAGG..G.TCCTG.	9	13
Onion	A...TTG....A.TGA.T...T.AAT.TC.A.....-.GG.AAG...T.G...	..TAT..AATG..G.TCCTG.	9	13
Ginger	A...TT..C..A.T.T.T.....AAT.CC.A.....-..G.AAG..CG.G...	..TAT..AATG..G.TCCTG.	8	13
Chilli	A...TTG....A.TGA.CGTT	T.AAT.CC.....-..G.AAG...C.G...	..TAT..AATG.GG.TCCCG.	12	14

Table 4.8: Pairwise distances of the beef cytb-specific 120 bp site against other 28 non-target species.

Species	Cow	Buffalo	Goat	Sheep	Deer	Donk	Horse	Pig	Dog	Cat	Rabb	Monk	Chik	Duck	Pegi	Quial	Rat	Salm	Tuna	Cord	Telap	Rohu	Pang	Frog	Turtl	Whet	Onion	Gingr	Chilli
Cow	0.000																												
Buffalo	0.220																												
Goat	0.144	0.145																											
Sheep	0.193	0.157	0.085																										
Deer	0.169	0.182	0.145	0.121																									
Donkey	0.197	0.237	0.158	0.196	0.240																								
Horse	0.239	0.212	0.197	0.250	0.254	0.075																							
Pig	0.299	0.269	0.214	0.269	0.227	0.240	0.225																						
Dog	0.213	0.212	0.240	0.252	0.209	0.239	0.213	0.253																					
Cat	0.298	0.255	0.224	0.252	0.225	0.214	0.200	0.255	0.211																				
Rabbit	0.250	0.294	0.260	0.232	0.279	0.337	0.326	0.324	0.298	0.357																			
Monkey	0.426	0.444	0.338	0.369	0.462	0.284	0.269	0.284	0.420	0.422	0.503																		
Chicken	0.382	0.322	0.382	0.414	0.371	0.400	0.386	0.437	0.382	0.495	0.382	0.573																	
Duck	0.352	0.325	0.390	0.355	0.402	0.360	0.413	0.467	0.354	0.414	0.402	0.475	0.242																
Pigeon	0.429	0.338	0.330	0.329	0.381	0.359	0.411	0.441	0.453	0.515	0.432	0.457	0.242	0.289															
Quial	0.346	0.337	0.317	0.315	0.285	0.404	0.426	0.372	0.339	0.389	0.379	0.511	0.211	0.213	0.200														
Rat	0.254	0.148	0.174	0.173	0.159	0.226	0.200	0.225	0.146	0.186	0.342	0.351	0.388	0.396	0.387	0.307													
Salmon	0.510	0.427	0.404	0.370	0.442	0.430	0.432	0.417	0.362	0.415	0.386	0.491	0.363	0.372	0.399	0.367	0.419												
Tuna	0.344	0.241	0.268	0.228	0.230	0.281	0.284	0.301	0.270	0.317	0.315	0.509	0.316	0.406	0.367	0.289	0.200	0.312											
Cord	0.419	0.365	0.444	0.392	0.428	0.380	0.434	0.574	0.300	0.402	0.402	0.657	0.456	0.442	0.485	0.416	0.327	0.295	0.295										
Tilapia	0.282	0.282	0.225	0.251	0.268	0.294	0.312	0.383	0.241	0.327	0.345	0.431	0.377	0.400	0.355	0.347	0.270	0.252	0.282	0.238									
Rohu	0.386	0.441	0.353	0.367	0.385	0.367	0.386	0.424	0.367	0.425	0.334	0.538	0.456	0.388	0.447	0.400	0.286	0.297	0.298	0.308	0.271								
Pangas	0.443	0.391	0.413	0.487	0.386	0.529	0.491	0.371	0.367	0.407	0.546	0.552	0.426	0.389	0.522	0.407	0.321	0.230	0.333	0.309	0.333	0.214							
Frog	0.357	0.314	0.323	0.267	0.345	0.313	0.345	0.380	0.333	0.336	0.342	0.398	0.474	0.428	0.414	0.398	0.269	0.422	0.355	0.328	0.373	0.297	0.352						
Turtle	0.362	0.471	0.408	0.426	0.389	0.411	0.445	0.450	0.393	0.380	0.489	0.494	0.421	0.367	0.415	0.331	0.378	0.430	0.429	0.341	0.374	0.327	0.317	0.331					
Wheat	1.858	1.863	1.888	1.682	1.975	1.915	2.066	2.424	1.801	1.955	1.631	2.020	2.323	2.171	2.001	2.172	1.708	1.899	1.842	1.830	1.815	1.815	2.221	1.817	2.229				
Onion	1.919	1.974	1.662	1.787	1.747	2.012	2.079	2.198	1.915	1.827	1.870	1.981	2.380	2.171	2.080	2.320	1.687	2.176	1.640	2.083	2.198	2.108	2.012	1.842	2.117	0.806			
Ginger	1.786	2.056	1.810	1.504	1.547	1.906	2.017	2.017	1.519	1.634	1.810	2.010	1.784	1.659	1.862	1.639	1.605	1.457	1.645	1.433	1.391	1.605	1.571	1.860	1.969	1.088	1.126		
Chilli	1.969	2.129	1.985	1.529	1.786	2.019	2.121	2.200	1.629	1.669	1.933	2.137	2.131	1.862	2.038	1.964	1.560	1.519	1.772	1.464	1.488	1.772	1.690	1.874	2.069	0.999	1.231	0.109	0.000

Table 4.9: Pairwise distances of the beef ND5-specific 106 bp site against other 28 non-target species.

Species	Cow	Buffa	Goat	Sheep	Deer	Donk	Horse	Pig	Dog	Cat	Rabb	Monk	Chik	Duck	Pegi	Quial	Rat	Salm	Tuna	Cord	Telap	Rohu	Pang	Frog	Turtl	Whet	Onion	Gingr	Chilli
Cow	0.000																												
Buffalo	0.185																												
Goat	0.311	0.145																											
Sheep	0.208	0.157	0.195																										
Deer	0.267	0.187	0.269	0.269																									
Donkey	0.392	0.338	0.358	0.333	0.461																								
Horse	0.424	0.353	0.338	0.348	0.478	0.097																							
Pig	0.471	0.351	0.351	0.329	0.461	0.408	0.461																						
Dog	0.514	0.397	0.402	0.421	0.479	0.448	0.402	0.426																					
Cat	0.559	0.455	0.397	0.432	0.455	0.435	0.405	0.351	0.297																				
Rabbit	0.492	0.435	0.482	0.306	0.555	0.327	0.329	0.492	0.435	0.499																			
Monkey	0.568	0.503	0.587	0.503	0.485	0.472	0.435	0.529	0.607	0.597	0.550																		
Chicken	0.776	0.623	0.643	0.644	0.572	0.654	0.572	0.602	0.452	0.540	0.598	0.752																	
Duck	0.680	0.500	0.612	0.563	0.607	0.563	0.482	0.654	0.563	0.622	0.627	0.752	0.202																
Pigeon	0.817	0.564	0.718	0.664	0.607	0.588	0.569	0.654	0.565	0.607	0.608	0.665	0.293	0.363															
Quail	0.752	0.612	0.676	0.602	0.633	0.654	0.553	0.572	0.500	0.486	0.540	0.816	0.171	0.216	0.327														
Rat	0.506	0.416	0.496	0.413	0.489	0.455	0.421	0.622	0.461	0.496	0.510	0.563	0.638	0.653	0.551	0.693													
Salmon	0.546	0.536	0.536	0.542	0.559	0.659	0.702	0.559	0.496	0.607	0.648	0.833	0.486	0.592	0.503	0.602	0.485												
Tuna	0.587	0.544	0.540	0.422	0.649	0.607	0.592	0.578	0.465	0.458	0.504	0.712	0.397	0.530	0.526	0.526	0.486	0.348											
Cord	0.696	0.622	0.549	0.522	0.638	0.709	0.778	0.622	0.680	0.536	0.649	1.093	0.602	0.587	0.627	0.607	0.622	0.507	0.526										
Tilapia	0.578	0.654	0.578	0.514	0.648	0.685	0.691	0.663	0.577	0.681	0.572	0.914	0.555	0.617	0.664	0.654	0.617	0.366	0.286	0.472									
Rohu	0.492	0.432	0.510	0.333	0.574	0.518	0.518	0.569	0.411	0.503	0.348	0.670	0.607	0.507	0.568	0.705	0.441	0.315	0.276	0.478	0.302								
Pangas	0.612	0.597	0.553	0.489	0.587	0.745	0.693	0.671	0.557	0.654	0.581	0.851	0.672	0.586	0.665	0.742	0.617	0.319	0.308	0.447	0.286	0.258							
Frog	0.955	1.054	1.116	0.977	0.955	0.944	0.887	0.973	0.735	0.695	0.824	1.102	0.974	1.230	0.925	0.955	0.973	1.047	0.851	1.015	1.041	0.807	0.815						
Turtle	0.768	0.693	0.676	0.562	0.735	0.659	0.693	0.544	0.458	0.617	0.644	0.781	0.338	0.402	0.373	0.419	0.607	0.522	0.405	0.622	0.602	0.562	0.676	0.907					
Wheat	1.993	1.961	2.012	1.801	1.989	1.974	2.064	1.971	1.638	1.637	1.961	2.328	1.998	2.328	2.140	2.117	1.775	1.878	2.011	2.271	2.059	1.723	1.850	1.776	1.874				
Onion	1.662	1.754	1.889	1.403	1.799	2.004	2.135	1.897	1.575	1.895	1.850	2.320	1.906	2.052	2.011	1.874	1.480	1.902	1.828	1.908	1.517	1.222	1.549	1.749	1.664	0.172			
Ginger	1.849	1.626	1.801	1.732	1.894	2.002	1.931	2.210	1.678	1.965	1.955	1.751	1.927	1.802	1.877	2.018	1.511	2.011	1.989	2.254	1.828	1.576	1.898	1.992	1.927	0.248	0.216		
Chilli	1.876	1.974	2.152	1.691	1.966	1.984	2.023	1.952	1.732	1.998	2.090	2.044	2.145	2.092	2.575	2.145	2.037	2.274	2.018	2.251	1.975	1.890	2.045	1.935	1.959	0.391	0.402	0.355	0.000

Table 4.10: Pairwise distances of the buffalo cytb-specific 90 bp site against other 28 non-target species.

Species	Buffa	cow	Goat	Sheep	Deer	Donk	Horse	Pork	Dog	Cat	Rabb	Monk	Chik	Duck	Pegi	Quial	Rat	Salm	Tuna	Cord	Telap	Rohu	Pang	Frog	Torts	Whet	Onion	Gingr	Chilli
Buffalo	0.000																												
Cow	0.244																												
Goat	0.277	0.255																											
Sheep	0.305	0.302	0.145																										
Deer	0.247	0.268	0.260	0.358																									
Donkey	0.385	0.302	0.334	0.350	0.368																								
Horse	0.331	0.268	0.236	0.264	0.379	0.132																							
Pig	0.260	0.218	0.283	0.315	0.250	0.347	0.296																						
Dog	0.353	0.287	0.253	0.267	0.330	0.310	0.248	0.316																					
Cat	0.332	0.270	0.267	0.266	0.384	0.303	0.280	0.267	0.264																				
Rabbit	0.401	0.339	0.298	0.300	0.312	0.318	0.314	0.429	0.327	0.303																			
Monkey	0.411	0.345	0.304	0.409	0.429	0.338	0.272	0.413	0.396	0.391	0.413																		
Chicken	0.377	0.307	0.308	0.415	0.437	0.439	0.359	0.321	0.377	0.399	0.477	0.440																	
Duck	0.408	0.352	0.273	0.383	0.420	0.424	0.283	0.350	0.413	0.406	0.428	0.434	0.161																
Pegion	0.409	0.274	0.356	0.433	0.393	0.486	0.415	0.374	0.455	0.420	0.479	0.396	0.208	0.253															
Quial	0.440	0.346	0.327	0.457	0.484	0.501	0.384	0.344	0.420	0.532	0.551	0.484	0.136	0.239	0.289														
Rat	0.441	0.379	0.223	0.271	0.297	0.401	0.366	0.433	0.325	0.397	0.285	0.429	0.520	0.444	0.510	0.571													
Salmon	0.452	0.409	0.433	0.422	0.522	0.451	0.494	0.374	0.429	0.301	0.620	0.532	0.430	0.554	0.615	0.498	0.545												
Tuna	0.448	0.354	0.379	0.407	0.483	0.471	0.433	0.337	0.496	0.389	0.500	0.524	0.305	0.356	0.511	0.378	0.480	0.302											
Cord	0.410	0.479	0.384	0.393	0.499	0.548	0.440	0.455	0.460	0.476	0.575	0.500	0.475	0.419	0.600	0.410	0.457	0.341	0.341										
Tilapia	0.452	0.462	0.267	0.279	0.474	0.561	0.452	0.435	0.412	0.374	0.473	0.532	0.464	0.539	0.600	0.462	0.397	0.236	0.359	0.236									
Rohu	0.451	0.397	0.284	0.328	0.476	0.592	0.522	0.399	0.454	0.336	0.553	0.455	0.509	0.435	0.511	0.511	0.371	0.340	0.286	0.254	0.270								
Pangas	0.442	0.348	0.391	0.485	0.513	0.532	0.525	0.442	0.459	0.366	0.613	0.470	0.517	0.548	0.489	0.629	0.539	0.269	0.306	0.359	0.304	0.238							
Frog	0.547	0.531	0.419	0.428	0.548	0.668	0.624	0.658	0.641	0.573	0.408	0.736	0.612	0.633	0.559	0.609	0.484	0.668	0.581	0.599	0.467	0.507	0.586						
Turtle	0.551	0.568	0.470	0.448	0.521	0.500	0.479	0.464	0.374	0.383	0.558	0.736	0.510	0.514	0.609	0.583	0.570	0.520	0.514	0.550	0.535	0.466	0.581	0.508					
Wheat	1.609	1.886	1.188	1.185	1.290	1.265	1.419	1.319	1.459	1.274	1.301	1.410	1.252	1.287	1.535	1.264	1.643	1.193	1.203	1.379	1.393	1.187	1.551	1.480	1.246				
Onion	1.280	1.459	0.935	0.927	1.173	1.319	1.280	1.149	1.117	1.002	1.178	1.221	1.013	1.042	1.227	1.115	1.335	1.055	1.030	1.115	1.131	0.964	1.270	1.346	1.008	0.095			
Ginger	1.333	1.525	0.970	0.963	1.173	1.265	1.131	1.104	1.163	1.040	1.178	1.272	1.013	1.042	1.227	1.115	1.396	1.099	1.071	1.162	1.178	1.002	1.322	1.480	1.051	0.094	0.046		
Chilli	1.380	1.583	1.003	0.996	1.315	1.555	1.384	1.205	1.172	1.046	1.327	1.376	1.060	1.120	1.322	1.171	1.519	1.107	1.078	1.171	1.187	1.008	1.333	1.543	0.973	0.160	0.057	0.081	0.000

Table 4.11: Pairwise distances of the buffalo ND5-specific 138 bp site against other 28 non-target species.

Species	Buffa	Cow	Goat	Sheep	Deer	Donk	Horse	Pig	Dog	Cat	Rabb	Monk	Chik	Duck	Pegi	Quial	Rat	Salm	Tuna	Cord	Telap	Rohu	Pang	Frog	Torts	Whet	Onion	Gingr	Chilli
Buffalo	0.000																												
Cow	0.175																												
Goat	0.239	0.166																											
Sheep	0.216	0.186	0.079																										
Deer	0.196	0.219	0.254	0.208																									
Donkey	0.421	0.378	0.443	0.415	0.453																								
Horse	0.435	0.406	0.446	0.418	0.426	0.079																							
Pigeon	0.308	0.283	0.347	0.310	0.285	0.390	0.362																						
Dog	0.414	0.421	0.484	0.452	0.386	0.447	0.436	0.461																					
Cat	0.440	0.485	0.484	0.411	0.435	0.385	0.417	0.302	0.347																				
Rabbit	0.608	0.596	0.646	0.604	0.616	0.655	0.651	0.576	0.757	0.671																			
Monkey	0.502	0.506	0.464	0.418	0.537	0.550	0.573	0.557	0.565	0.645	0.534																		
Chicken	1.116	1.113	1.007	0.914	0.951	0.879	0.836	1.078	1.192	0.940	0.951	0.879																	
Duck	1.066	1.264	1.319	1.221	1.329	1.022	0.884	1.376	1.298	1.048	0.976	1.008	0.447																
Pigeon	1.000	1.039	1.133	1.040	0.978	0.872	0.796	0.977	0.951	0.964	0.926	0.818	0.296	0.466															
Quail	1.178	1.176	1.180	1.066	1.106	0.811	0.771	1.052	1.138	0.972	0.849	0.817	0.219	0.471	0.374														
Rat	0.535	0.537	0.531	0.521	0.497	0.675	0.620	0.495	0.496	0.592	0.655	0.601	0.903	1.178	0.875	0.882													
Salmon	0.900	1.049	0.812	0.740	0.900	0.923	0.909	1.037	1.238	0.973	1.088	0.869	0.871	1.010	0.832	0.880	1.092												
Tuna	0.992	1.000	0.796	0.793	0.989	0.850	0.774	0.995	0.982	0.867	0.964	0.690	0.796	0.771	0.756	0.812	0.827	0.465											
Cord	0.894	0.956	0.751	0.751	0.950	1.016	0.917	0.913	1.025	0.977	0.946	0.638	0.748	1.105	0.844	0.903	0.778	0.506	0.435										
Tilapia	0.738	0.803	0.802	0.751	0.836	0.940	0.850	0.766	0.976	0.846	1.023	0.776	0.605	0.774	0.654	0.639	0.775	0.510	0.325	0.489									
Rohu	1.130	0.922	0.778	0.781	0.889	0.881	0.860	0.903	1.133	0.852	0.925	0.770	0.859	1.143	0.819	0.812	0.963	0.564	0.632	0.681	0.758								
Pangas	1.091	1.035	0.999	0.874	0.905	1.003	1.011	0.918	0.922	0.906	1.166	0.944	0.772	1.125	0.721	0.822	0.979	0.766	0.683	0.808	0.632	0.621							
Frog	1.466	1.356	1.260	1.246	1.334	1.211	1.166	1.514	1.518	1.426	1.705	1.294	1.297	1.538	1.526	1.329	1.220	1.368	1.263	1.154	1.708	1.796	1.263						
Turtle	0.913	0.870	0.813	0.747	0.917	0.759	0.752	0.846	0.871	0.739	0.723	0.889	0.600	0.680	0.698	0.614	0.824	0.787	0.655	0.840	0.750	0.732	0.824	1.678					
Wheat	1.663	1.563	1.590	1.747	1.950	1.924	2.224	1.981	2.128	2.089	3.245	2.306	2.166	3.358	2.881	2.777	1.640	2.320	3.264	3.156	2.146	3.091	2.954	2.089	2.899				
Onion	1.686	1.602	1.705	1.739	1.866	1.949	2.102	2.195	2.078	2.048	2.948	2.165	2.180	3.255	2.152	2.056	1.610	2.890	2.961	3.229	2.337	2.104	3.194	1.970	2.915	0.139			
Ginger	1.441	1.399	1.423	1.377	1.622	1.583	1.663	1.854	1.798	2.136	2.087	2.002	2.163	2.757	2.415	2.179	1.241	2.322	2.296	2.322	2.133	2.123	2.981	1.949	2.107	0.159	0.159		
Chilli	1.472	1.590	1.634	1.694	1.671	1.844	1.513	2.206	2.147	2.249	2.050	1.943	2.240	2.251	2.157	2.029	1.350	2.011	2.320	2.994	2.015	2.036	2.981	2.036	2.185	0.216	0.250	0.252	0.000

Table 4.12: Pairwise distances of the pork cytb-specific 146 bp site against other 28 non-target species.

Species	Pig	Buffa	Cow	Goat	Sheep	Deer	Donk	Horse	Dog	Cat	Rabb	Monk	Chik	Duck	Pige	Quial	Rat	Salm	Tuna	Cord	Telap	Rohu	Pang	Frog	Turtl	Whet	Onion	Gingr	Chilli
Pig	0.000																												
Buffalo	0.291																												
Cow	0.283	0.163																											
Goat	0.206	0.155	0.138																										
Sheep	0.262	0.172	0.181	0.088																									
Deer	0.252	0.217	0.190	0.172	0.122																								
Donkey	0.262	0.231	0.194	0.193	0.212	0.253																							
Horse	0.261	0.240	0.250	0.239	0.249	0.223	0.081																						
Dog	0.220	0.240	0.233	0.243	0.272	0.279	0.290	0.259																					
Cat	0.254	0.240	0.262	0.231	0.249	0.241	0.251	0.232	0.220																				
Rabbit	0.320	0.288	0.249	0.236	0.227	0.279	0.320	0.299	0.272	0.341																			
Monkey	0.249	0.359	0.330	0.284	0.315	0.373	0.283	0.262	0.348	0.348	0.426																		
Chicken	0.443	0.340	0.349	0.359	0.370	0.363	0.384	0.383	0.408	0.450	0.345	0.477																	
Duck	0.507	0.353	0.361	0.384	0.362	0.372	0.406	0.430	0.428	0.472	0.382	0.420	0.223																
Pegion	0.406	0.401	0.445	0.360	0.371	0.410	0.405	0.452	0.465	0.503	0.431	0.434	0.203	0.244															
Quial	0.384	0.362	0.360	0.339	0.350	0.349	0.413	0.424	0.388	0.417	0.395	0.455	0.176	0.213	0.167														
Rat	0.210	0.186	0.255	0.196	0.224	0.221	0.244	0.242	0.191	0.232	0.346	0.328	0.372	0.430	0.385	0.342													
Salmon	0.393	0.418	0.450	0.394	0.371	0.434	0.414	0.414	0.379	0.404	0.405	0.425	0.380	0.422	0.379	0.383	0.358												
Tuna	0.315	0.271	0.314	0.232	0.232	0.263	0.290	0.290	0.303	0.323	0.300	0.442	0.315	0.401	0.387	0.309	0.222	0.270											
Cord	0.533	0.410	0.440	0.443	0.387	0.446	0.424	0.435	0.323	0.456	0.430	0.632	0.480	0.495	0.517	0.468	0.341	0.288	0.319										
Tilapia	0.415	0.305	0.326	0.281	0.302	0.305	0.333	0.301	0.286	0.349	0.359	0.409	0.378	0.408	0.402	0.395	0.296	0.281	0.302	0.268									
Rohu	0.412	0.392	0.366	0.368	0.391	0.441	0.396	0.435	0.361	0.364	0.362	0.491	0.455	0.428	0.450	0.389	0.315	0.320	0.314	0.378	0.352								
Pangas	0.381	0.399	0.463	0.431	0.507	0.423	0.526	0.469	0.367	0.475	0.464	0.502	0.437	0.422	0.472	0.433	0.334	0.281	0.349	0.352	0.398	0.262							
Frog	0.398	0.333	0.365	0.364	0.322	0.402	0.348	0.359	0.312	0.384	0.356	0.406	0.441	0.463	0.444	0.392	0.345	0.409	0.376	0.365	0.430	0.349	0.358						
Turtle	0.418	0.407	0.316	0.358	0.369	0.343	0.358	0.366	0.366	0.398	0.402	0.377	0.385	0.336	0.415	0.357	0.336	0.427	0.381	0.391	0.355	0.355	0.357	0.336					
Wheat	1.574	1.655	1.904	1.771	1.779	1.697	2.276	2.114	1.915	1.795	1.557	1.482	2.583	2.137	2.564	2.731	1.736	2.261	1.989	2.035	2.194	2.237	2.082	1.676	2.186				
Onion	1.430	1.671	1.951	1.701	1.709	1.626	1.884	1.823	1.875	1.544	1.495	1.261	2.083	1.914	2.269	2.165	1.384	1.844	1.600	1.922	2.101	2.059	1.795	1.607	1.926	0.138			
Ginger	1.569	1.619	1.930	1.840	1.744	1.613	2.162	1.864	1.779	1.568	1.522	1.379	2.180	2.034	2.187	2.253	1.522	2.204	1.905	1.915	2.264	1.943	1.908	1.668	1.834	0.167	0.175		
Chilli	1.600	1.510	1.944	1.786	1.740	1.601	1.998	1.737	1.914	1.593	1.549	1.319	2.315	2.158	2.227	2.187	1.493	2.028	1.678	1.720	1.871	1.797	1.775	1.519	1.890	0.266	0.246	0.312	0.000

Table 4.13: Pairwise distances of the pork ND5-specific 73 bp site against other 28 non-target species.

Species	Pig	Buffalo	Cow	Goat	Sheep	Deer	Donk	Horse	Dog	Cat	Rabb	Monk	Chik	Duck	Pegi	Quial	Rat	Salm	Tuna	Cord	Telap	Rohu	Pang	Frog	Turtl	Whet	Onion	Gingr	Chilli
Pig	0.000																												
Buffalo	0.395																												
Cow	0.483	0.170																											
Goat	0.374	0.107	0.255																										
Sheep	0.365	0.165	0.144	0.186																									
Deer	0.595	0.214	0.260	0.281	0.280																								
Donkey	0.388	0.317	0.380	0.356	0.319	0.470																							
Horse	0.435	0.313	0.374	0.376	0.314	0.462	0.069																						
Dog	0.447	0.418	0.663	0.397	0.490	0.528	0.590	0.520																					
Cat	0.421	0.557	0.706	0.449	0.525	0.518	0.621	0.547	0.337																				
Rabbit	0.527	0.490	0.572	0.575	0.410	0.626	0.375	0.344	0.518	0.661																			
Monkey	0.573	0.566	0.647	0.583	0.563	0.573	0.679	0.598	0.432	0.410	0.652																		
Chicken	0.822	0.962	1.398	0.935	1.013	0.796	1.040	0.916	0.685	0.755	0.639	0.755																	
Duck	0.739	0.682	0.902	0.759	0.707	0.831	0.662	0.616	0.747	0.826	0.604	0.822	0.238																
Pigeon	0.882	0.735	1.069	1.034	0.882	0.817	0.803	0.750	0.705	0.851	0.582	0.929	0.325	0.393															
Quail	0.782	0.871	1.122	0.982	0.909	0.974	0.891	0.748	0.715	0.621	0.607	0.715	0.212	0.196	0.334														
Rat	0.641	0.530	0.635	0.567	0.464	0.601	0.632	0.501	0.472	0.507	0.539	0.530	0.782	0.816	0.612	0.782													
Salmon	0.606	0.674	0.582	0.564	0.552	0.734	0.947	0.965	0.572	0.651	0.592	0.789	0.693	0.866	0.775	0.755	0.630												
Tuna	0.696	0.879	0.857	0.772	0.679	0.902	0.885	0.909	0.539	0.578	0.581	0.593	0.531	0.797	0.675	0.662	0.526	0.262											
Cord	0.800	0.831	0.851	0.682	0.641	0.969	0.939	0.958	0.846	0.654	0.714	0.858	0.776	0.710	0.987	0.826	0.763	0.565	0.514										
Tilapia	0.727	0.779	0.727	0.654	0.607	0.876	0.822	0.928	0.701	0.857	0.634	0.652	0.692	0.746	0.855	0.863	0.668	0.317	0.238	0.480									
Rohu	0.620	0.632	0.629	0.626	0.466	0.758	0.731	0.682	0.510	0.600	0.391	0.492	0.690	0.658	0.711	0.739	0.589	0.258	0.242	0.413	0.241								
Pangas	0.722	0.707	0.658	0.589	0.485	0.797	0.782	0.764	0.647	0.647	0.547	0.501	0.838	0.739	1.039	0.865	0.643	0.263	0.217	0.362	0.239	0.172							
Frog	1.141	1.356	1.188	1.448	1.245	1.214	1.137	1.053	0.879	0.737	1.002	0.782	1.269	1.737	0.939	1.303	1.252	1.325	0.882	1.168	1.349	0.822	0.920						
Turtle	0.641	0.885	0.985	0.902	0.744	0.974	0.782	0.885	0.652	0.718	0.648	0.842	0.387	0.439	0.355	0.497	0.545	0.664	0.430	0.677	0.762	0.672	0.811	1.137					
Wheat	0.856	0.992	1.107	0.992	1.010	1.301	1.502	1.432	1.168	1.139	1.382	1.546	2.264	2.174	1.681	2.002	1.207	1.453	1.753	1.861	2.469	1.597	1.775	1.635	1.624				
Onion	0.815	0.937	0.974	0.937	0.955	1.127	1.371	1.258	0.992	1.117	1.156	1.441	2.276	1.982	1.702	1.873	1.076	1.282	1.595	1.709	2.207	1.453	1.615	1.913	1.542	0.126			
Ginger	0.691	0.741	0.934	0.741	0.810	0.981	1.097	1.019	0.737	0.969	1.091	1.095	1.666	1.427	1.386	1.493	0.932	1.107	1.307	1.694	1.860	1.237	1.388	2.074	1.380	0.196	0.170		
Chilli	0.912	0.919	1.063	0.981	0.999	1.354	1.332	1.226	1.018	1.196	1.183	1.356	2.150	1.927	1.636	2.019	1.036	1.278	1.472	1.701	2.074	1.196	1.578	1.642	1.542	0.171	0.167	0.263	0.000

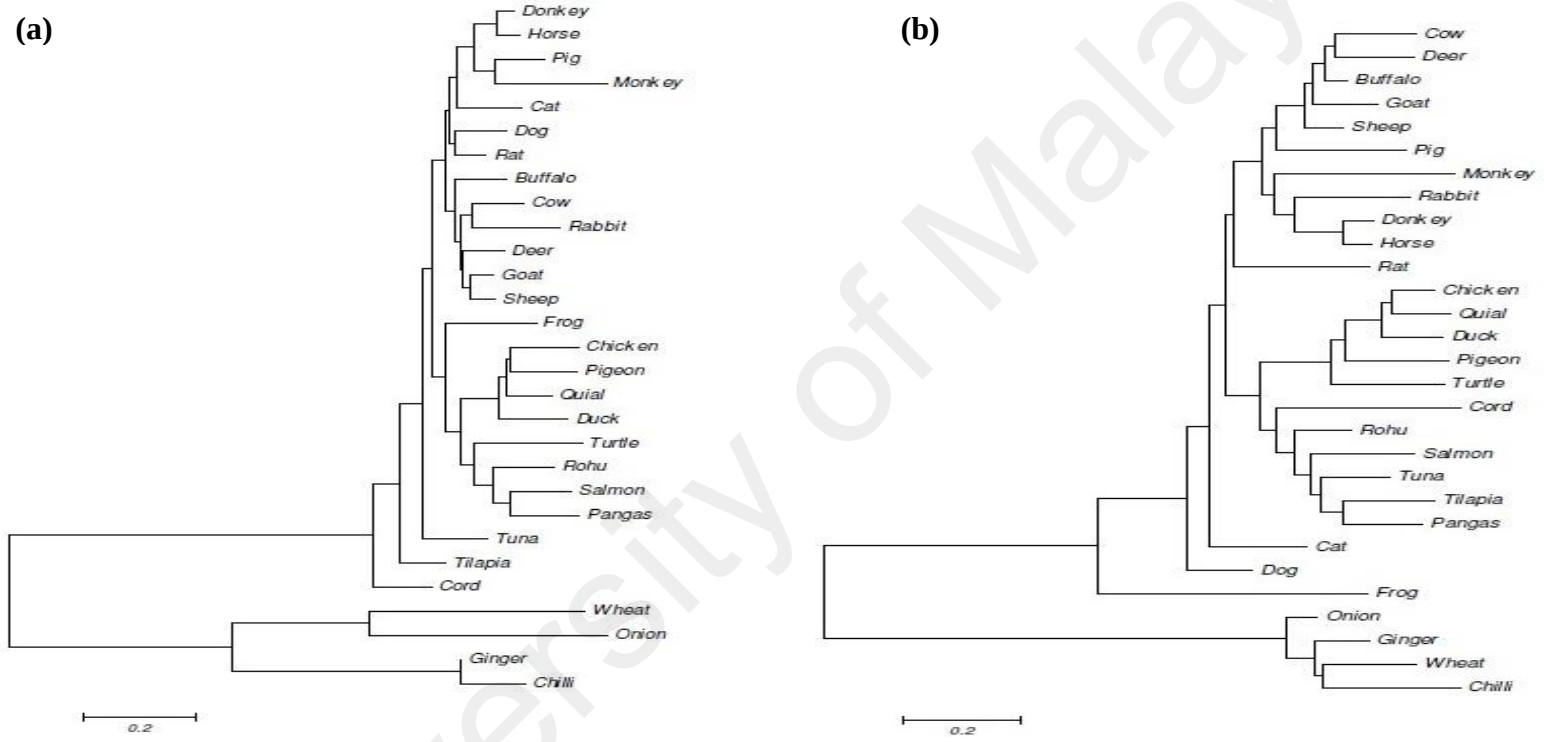


Figure 4.1: Phylogenetic tree generated from the amplicon sequences of each target gene and same gene sequences of other 28 non-target animal, aquatic and plant species using the neighbourhood-joining method. Phylogenetic tree of beef cytb-specific 120 bp site (a), beef ND5-specific 106 bp site (b), buffalo cytb-specific 90 bp site (c), buffalo ND5-specific 138 bp site (d), pork cytb-specific 146 bp site (e) and pork ND5-specific 106 bp site (f), respectively.

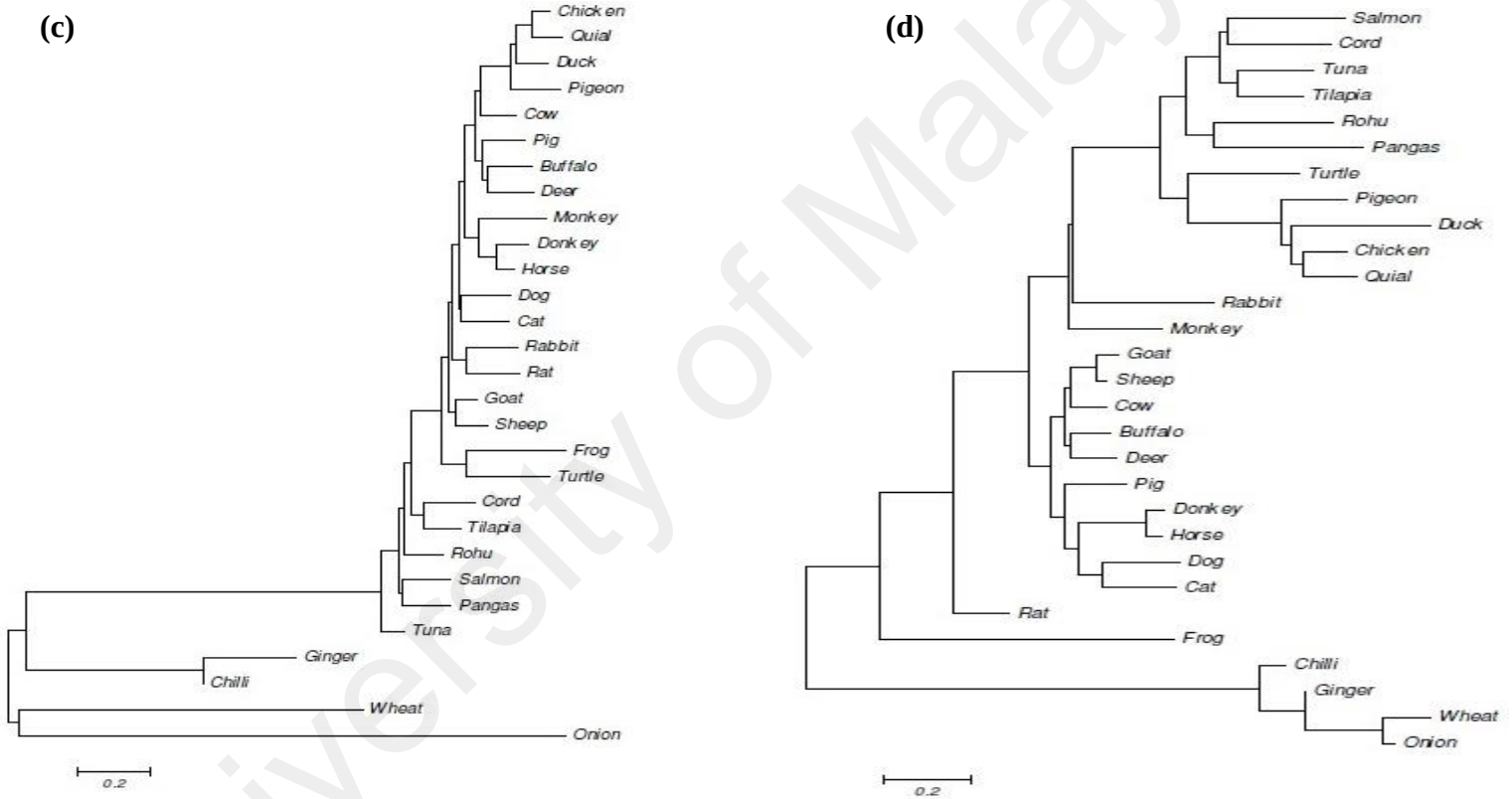


Figure 4.1: continued.

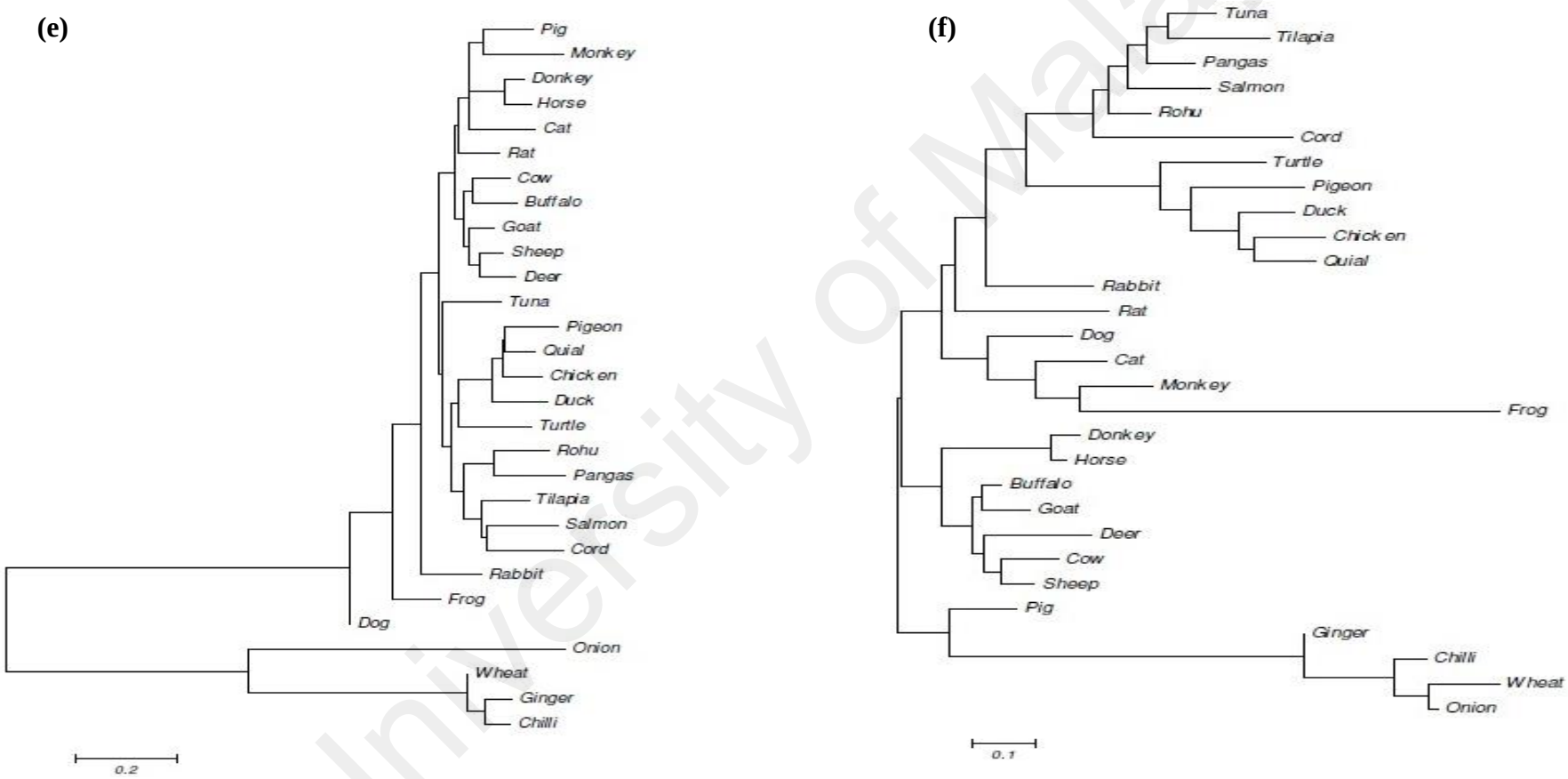


Figure 4.1: **c**ontinued.

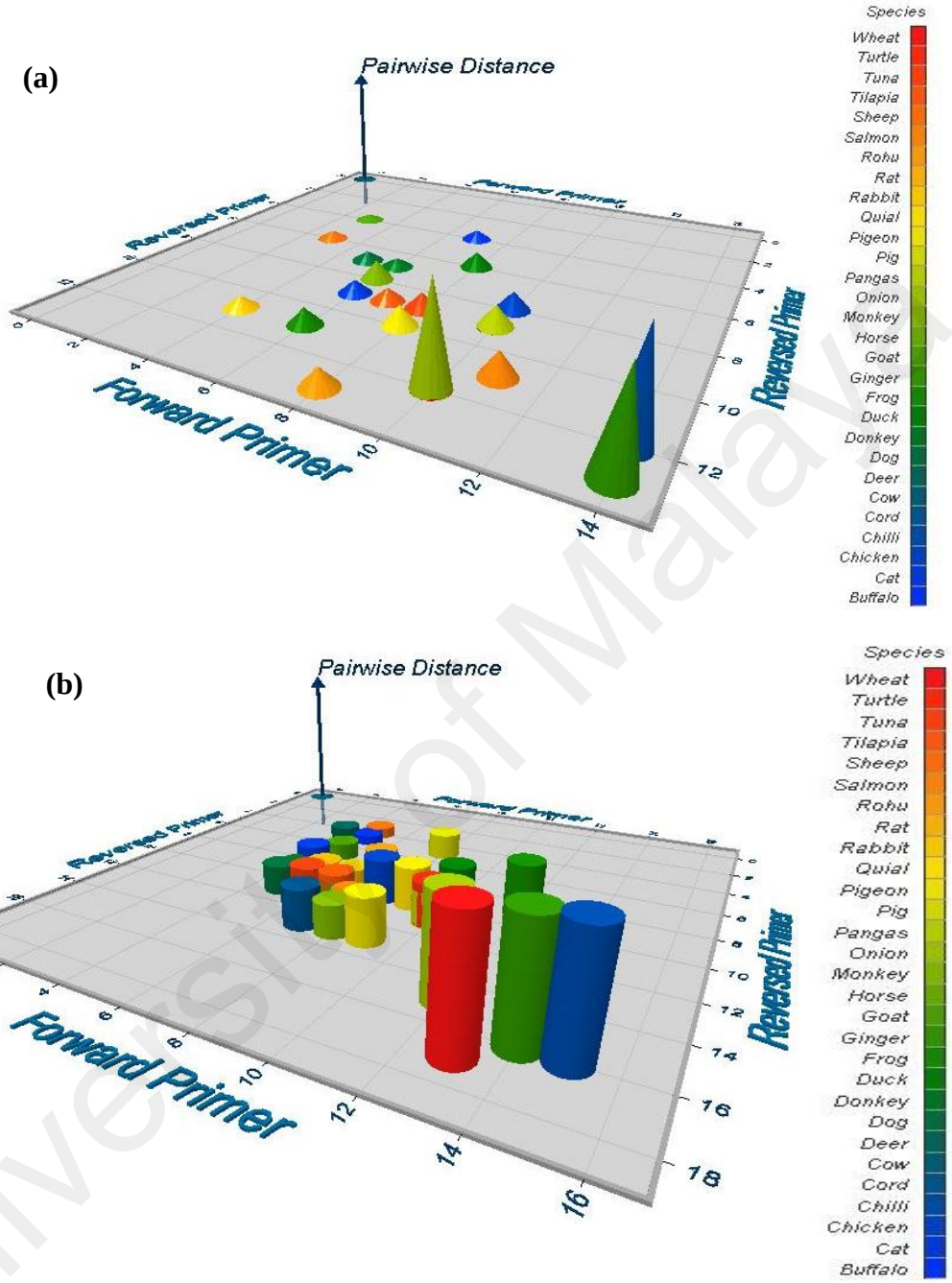


Figure 4.2: 3D plot showing mismatch and pairwise distance between targets and non-targets species. Here, X and Y axes represent the number of forward and reverse primer mismatches and Z axis represents pairwise distance between targets and other 28 non-target species. 3D plot from cytb and ND5 primer pairs are shown in figure (a) and (b) for cow; (c) and (d) for buffalo and (e) and (f) for pig, respectively.

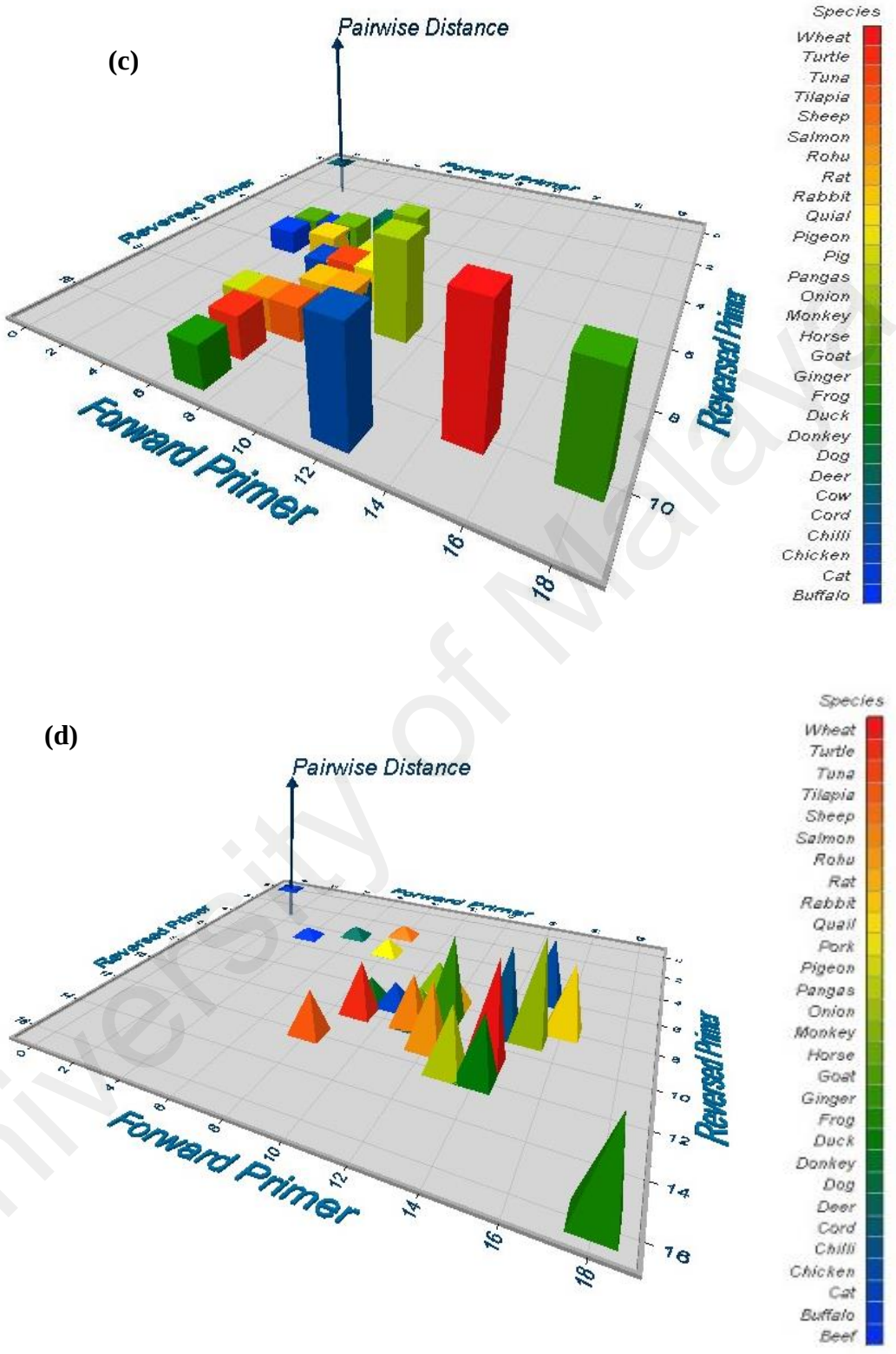


Figure 4.2: continued.

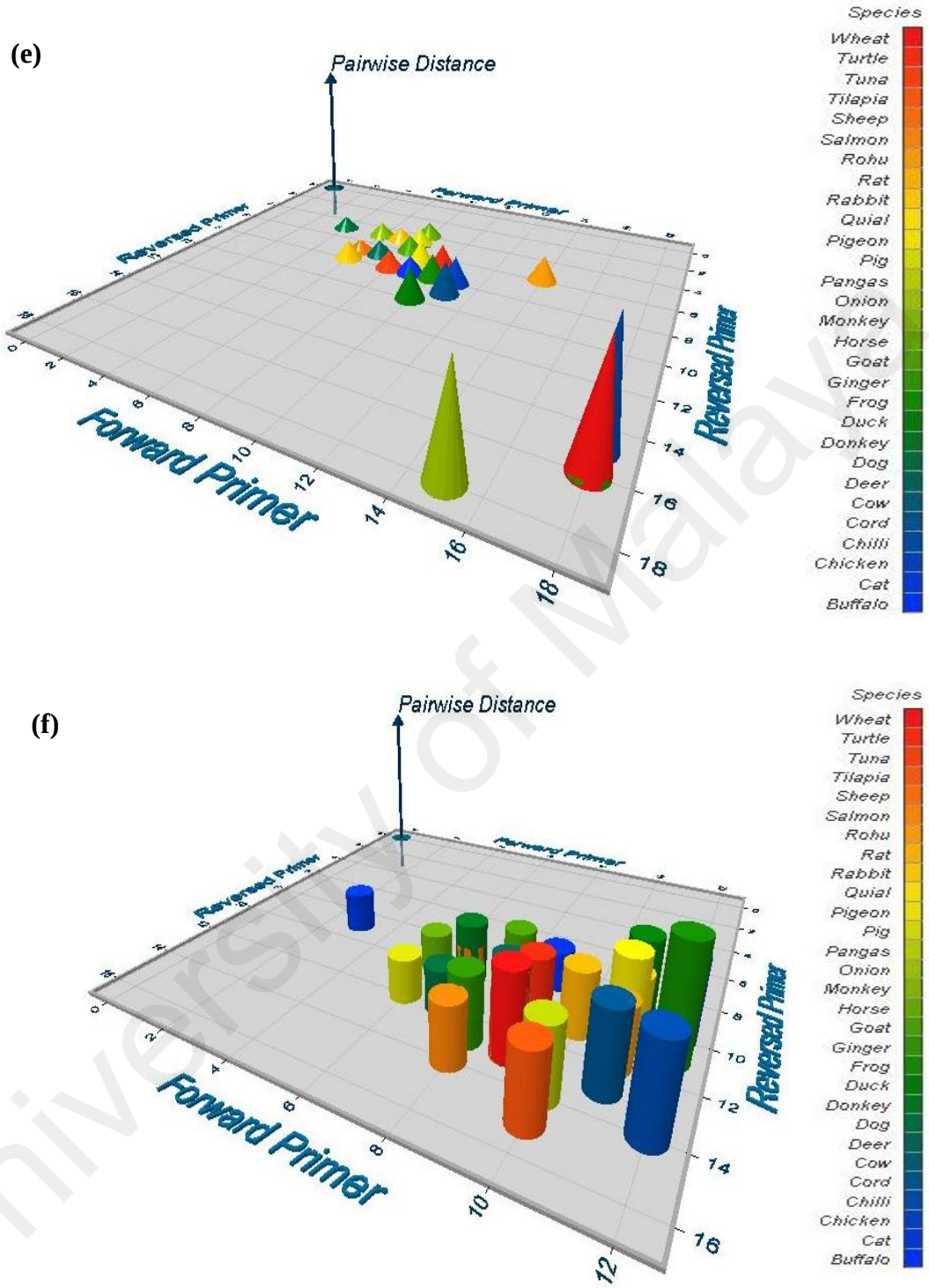


Figure 4.2: continued.

4.3 Simplex PCR Assay

4.3.1 Simplex PCR Optimization

To optimize the assays, the PCR reactions of six sets of primers were individually carried out on a gradient thermal cycler with total reaction volume of 25 µL containing appropriate quantity of all PCR components (section 3.4.1). The annealing temperatures of all sets of primers were checked from 58-62^0 C in the gradient system to find out the optimum annealing temperature for successful PCR amplifications. Although some primer sets were successfully amplified at both 59, 60 and 61^0 C, but were properly amplified only at 60^0 C (Figure 4.3 -4.5). Therefore, 60^0 C temperature was the optimum annealing temperature for all the primer sets as in multiplex PCR reaction all primer pairs have to be amplified in a single reaction condition.

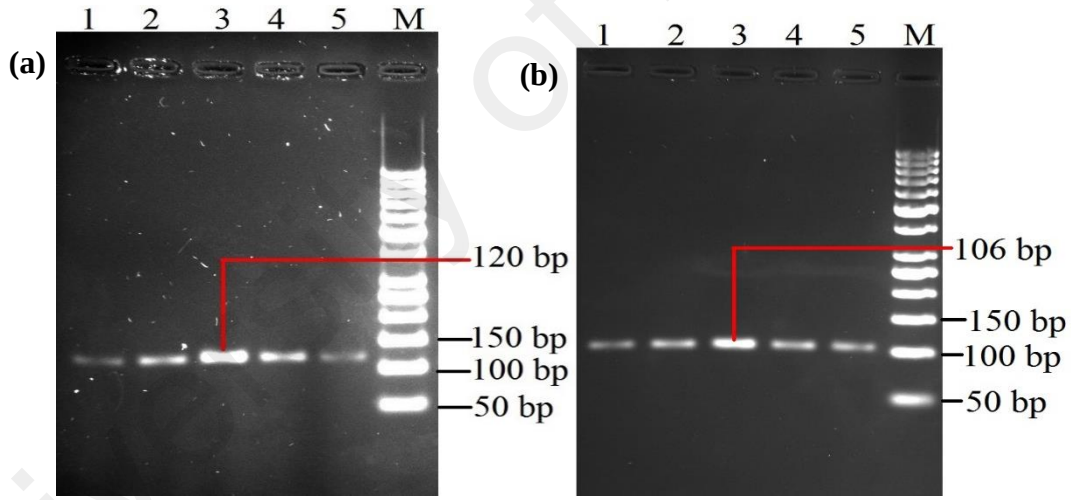


Figure 4.3: Optimization of annealing temperature of designed beef cytb (a) and ND5 (b) primer sets. In the gel image, M 50 bp DNA ladder; *lanes 1-5*, amplified PCR products for 58, 59, 60, 61 and 62^0 C temperatures.

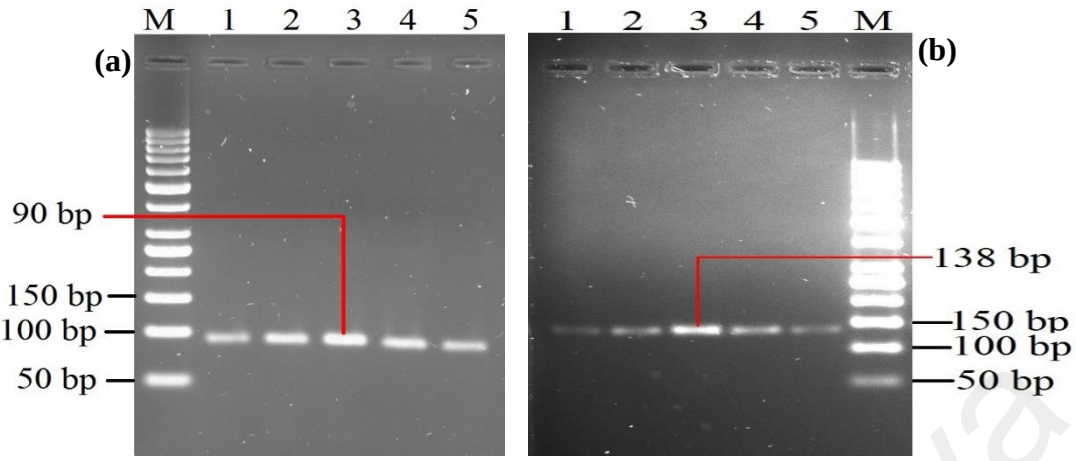


Figure 4.4: Optimization of annealing temperature of designed buffalo cytb (a) and ND5 (b) primer sets. In the gel image, M 50 bp DNA ladder; *lanes 1-5*, amplified PCR products for 58, 59, 60, 61 and 62^0 C temperatures.

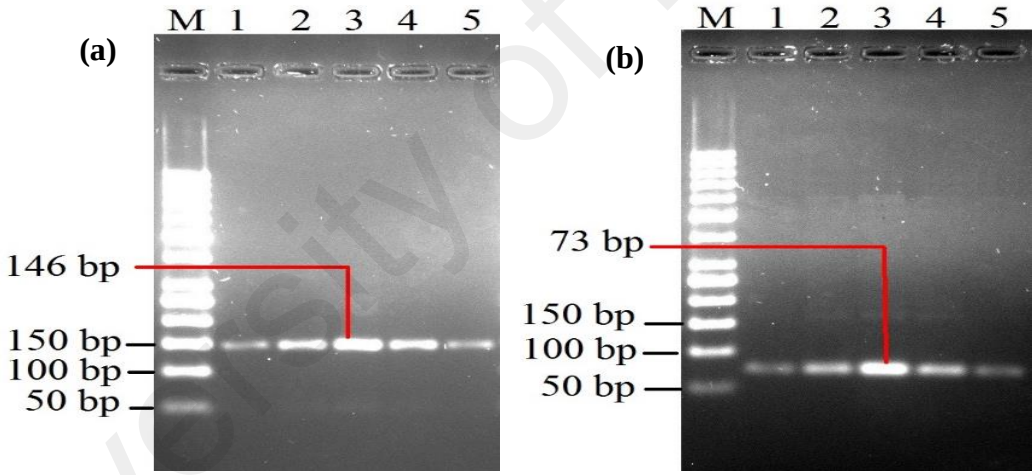


Figure 4.5: Optimization of annealing temperature of designed pork cytb (a) and ND5 (b) primer sets. In the gel image, M 50 bp DNA ladder; *lanes 1-5*, amplified PCR products for 58, 59, 60, 61 and 62^0 C temperatures.

4.3.2 Simplex PCR Assay Specificity

The specificity of the primers is very important in developing a robust PCR assay since the primers that fully match the target species and mismatch the non-target species offer

a higher chance of having a highly specific-PCR assay by eliminating the probability of non-target amplification (Wu, Hong & Liu, 2009).

After optimization of simplex PCR, species specificity of the primers were cross-tested against one target and other 22 non-target of terrestrial and aquatic animal species (beef, buffalo, pork, goat, lamb, dog, cat, rabbit, monkey, donkey, chicken, duck, pigeon, quail, rat, salmon, tuna, cod, tilapia, rohu, pangas, frog and tortoise) and 5 plant species (wheat, onion, garlic, ginger and pepper) which are commonly used as food matrices (Figure 4.6 - 4.11). The results showed that specific primer sets amplified only DNA of the target species but not any of the non-target species. While, universal eukaryotic primers amplified 99 bp sites from all species, reflecting the good quality of the extracted DNA and eliminating the possibility of any false-negative detection. This indicated a high specificity and fidelity of the each set of designed primers for the target species. All tests were repeated three times on three different days but the same outcomes were observed. The amplified PCR products were separated by running with automated capillary electrophoretic system (QIAGEN GmbH, Hilden, Germany). The system is capable of separating nucleic acid with 3-5 bp resolution by using an in-built gel cartridge within 12 min through the application of an electrical current to a gel-filled capillary cartridge via individual electrode of each capillary. The migrated nucleotide molecules were detected in an in-built-detector and displayed as gel image (Figure 4.6 - 4.11).

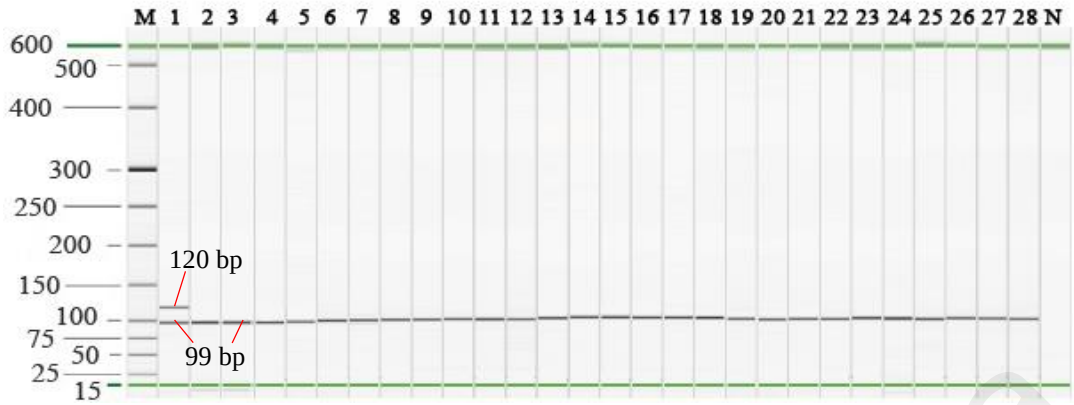


Figure 4.6: The specificity of the simplex PCR of beef cytb (120 bp)-specific primer pair with DNA of different species. In the gel image, M DNA ladder; N negative template control; *lane 1*, PCR products of beef cytb (120 bp) and endogenous control (99 bp); *lanes 2-28*, PCR products of endogenous control (99 bp) for buffalo, pork, goat, lamb, dog, cat, rabbit, monkey, donkey, chicken, duck, pigeon, quail, rat, salmon, tuna, cod, tilapia, rohu, pangas, frog, tortoise, wheat, onion, garlic, ginger and pepper, respectively.

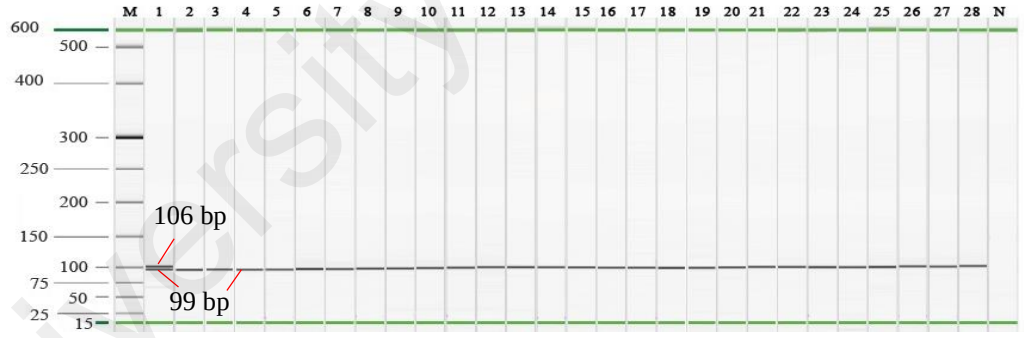


Figure 4.7: The specificity of the simplex PCR of beef ND5 (106 bp)-specific primer pair with DNA of different species. In the gel image, M DNA ladder; N negative template control; *lane 1*, PCR products of beef ND5 (106 bp) and endogenous control (99 bp); *lanes 2-28*, PCR products of endogenous control (99 bp) for buffalo, pork, goat, lamb, dog, cat, rabbit, monkey, donkey, chicken, duck, pigeon, quail, rat, salmon, tuna, cod, tilapia, rohu, pangas, frog, tortoise, wheat, onion, garlic, ginger and pepper, respectively.

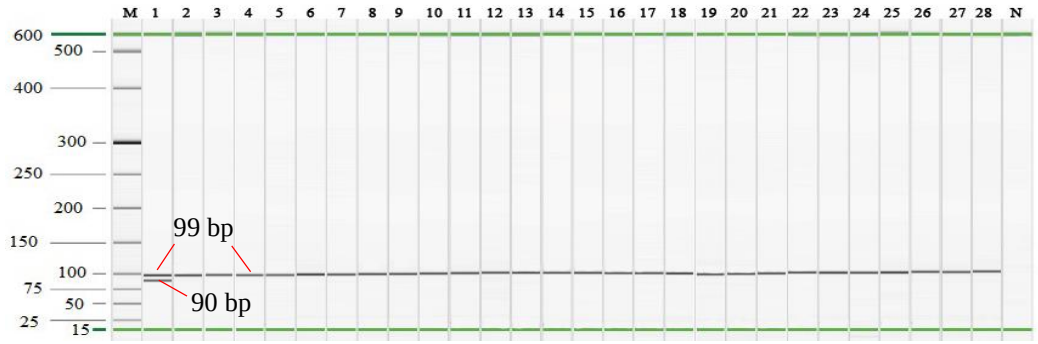


Figure 4.8: The specificity of the simplex PCR of buffalo cytb (90 bp)-specific primer pair with DNA of different species. In the gel image, M DNA ladder; N negative template control; *lane 1*, PCR products of buffalo cytb (90 bp) and endogenous control (99 bp); *lanes 2-28*, PCR products of endogenous control (99 bp) for beef, pork, goat, lamb, dog, cat, rabbit, monkey, donkey, chicken, duck, pigeon, quail, rat, salmon, tuna, cod, tilapia, rohu, pangas, frog, tortoise, wheat, onion, garlic, ginger and pepper, respectively.

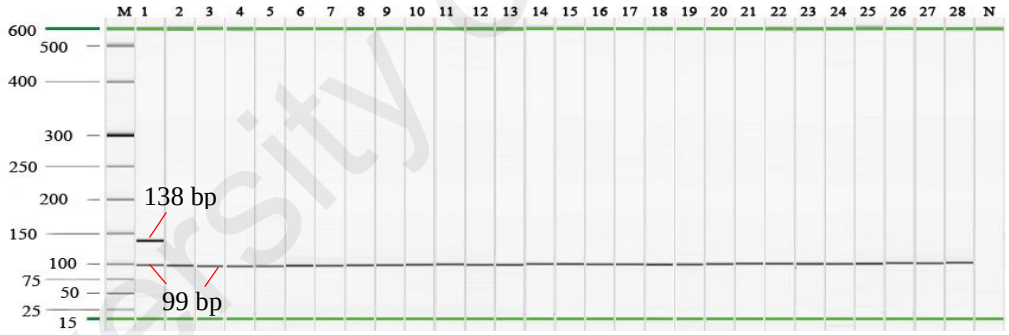


Figure 4.9: The specificity of the simplex PCR of buffalo ND5 (138 bp)-specific primer pair with DNA of different species. In the gel image, M DNA ladder; N negative template control; *lane 1*, PCR products of buffalo ND5 (138 bp) and endogenous control (99 bp); *lanes 2-28*, PCR products of endogenous control (99 bp) for beef, pork, goat, lamb, dog, cat, rabbit, monkey, donkey, chicken, duck, pigeon, quail, rat, salmon, tuna, cod, tilapia, rohu, pangas, frog, tortoise, wheat, onion, garlic, ginger and pepper, respectively.

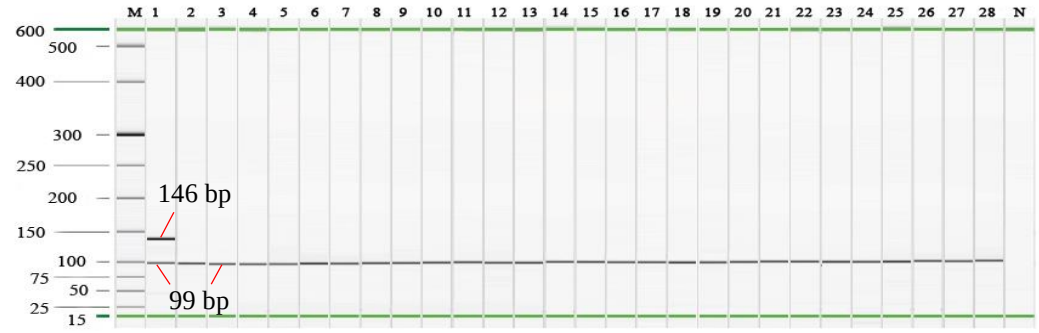


Figure 4.10: The specificity of the simplex PCR of pork cytb (146 bp)-specific primer pair with DNA of different species. In the gel image, M DNA ladder; N negative template control; *lane 1*, PCR products of pork cytb (146 bp) and endogenous control (99 bp); *lanes 2-28*, PCR products of endogenous control (99 bp) for beef, buffalo, goat, lamb, dog, cat, rabbit, monkey, donkey, chicken, duck, pigeon, quail, rat, salmon, tuna, cod, tilapia, rohu, pangas, frog, tortoise, wheat, onion, garlic, ginger and pepper, respectively.

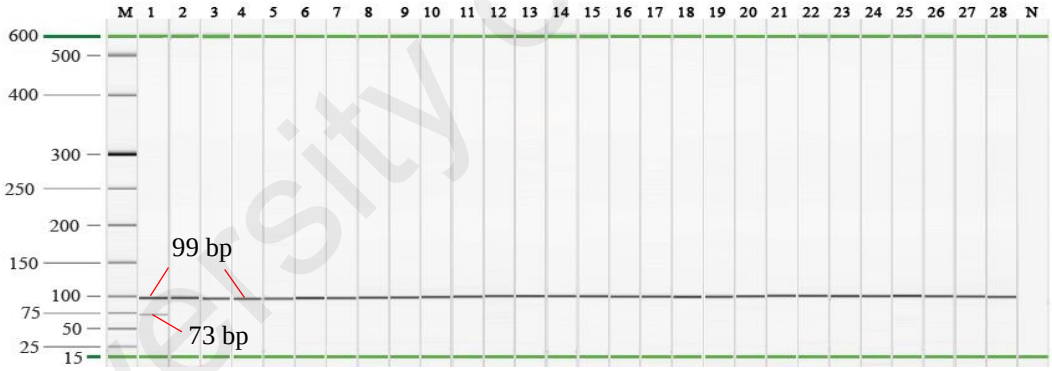


Figure 4.11: The specificity of the simplex PCR of pork ND5 (73 bp)-specific primer pair with DNA of different species. In the gel image, M DNA ladder; N negative template control; *lane 1*, PCR products of pork ND5 (73 bp) and endogenous control (99 bp); *lanes 2-28*, PCR products of endogenous control (99 bp) for beef, buffalo, goat, lamb, dog, cat, rabbit, monkey, donkey, chicken, duck, pigeon, quail, rat, salmon, tuna, cod, tilapia, rohu, pangas, frog, tortoise, wheat, onion, garlic, ginger and pepper, respectively.

4.3.3 PCR product sequencing

PCR products were sequenced to confirm the originality of the amplified PCR products of all targets. The obtained sequences were aligned firstly, with GenBank (www.ncbi.nlm.nih.gov) sequences for checking if there were matches and secondly, with specific gene sequence using the MEGA5 alignment tool to determine the similarity. The results of sequencing are summarized in Table 4.14. The sequence similarity score obtained from PCR products of beef (Cocytb and CoND5), Buffalo (Bucytb) and pork (Pocytb and PoND5) revealed 100% homology with *Bos taurus, Bubalus bubalis* and *Sus scrofa* sequences available in GenBank, respectively. On the contrary, only the PCR products of BuND5 showed the similarity of 98.55% with the ND5 gene of *Bubalus bubalis* but it was within acceptable range because the limit of sequence similarity for the designate species identification is at least 98% (Cawthorn et al., 2013).

Table 4.14: Sequencing results of PCR products.

Name of target	**Target gene**	**Species**	**GeneBank accession ID**	**Similarity (%)**
Cocytb	Cytb	Cow (*Bos taurus*)	V00654.1	100
CoND5	ND5	Cow (*Bos taurus*)	V00654.1	100
Bucytb	Cytb	Buffalo (*Bubalus bubalis*)	NC_006295.1	100
BuND5	ND5	Buffalo (*Bubalus bubalis*)	NC_006295.1	98.55
Pocytb	Cytb	Pork (*Sus scrofa*)	AF034253.1	100
PoND5	ND5	Pork (*Sus scrofa*)	AF034253.1	100

4.4 Tetraplex PCR Assay

4.4.1 Optimization of Tetraplex PCR Assay

Initially, simplex PCR was optimized for each primer pair against the template DNA extracted from muscle tissues of each target species to ensure the specificity and ability for amplifying the target sites of the designed primers (Dalmasso et al., 2004). The step by step development of a tetraplex PCR is demonstrated in Figure 4.12. As described in the methodology (Section 3.4.1 and 3.5.1), simplex (lanes 1-4), duplex (Lanes 5 and 6), triplex (lane 7) and the tetraplex (lane 8) PCR system were developed in an ordered way to ensure the clarity of the system (Ali et al., 2015d). The developed simplex, duplex, triplex and tetraplex systems amplified the targeted gene (Cytb and ND5) sites of fragment-size 120 and 106 bp for beef and 90 and 138 bp for buffalo, respectively (Figure 4.12), reflecting full consistency with the simplex PCR system.

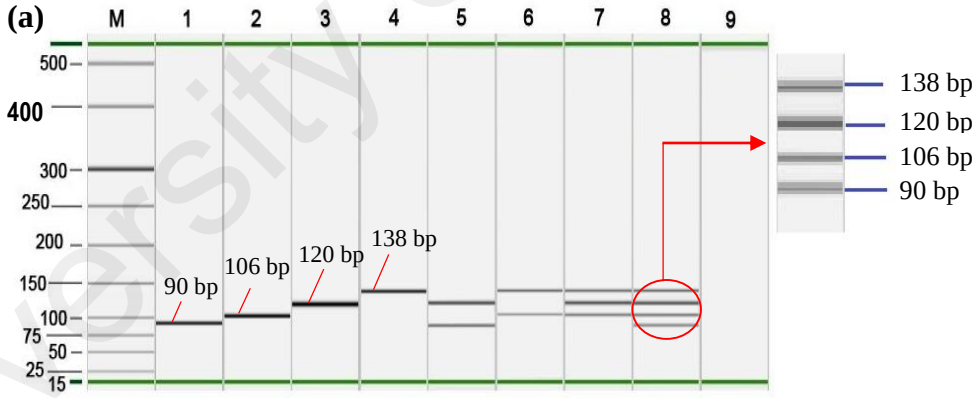


Figure 4.12: The gel image (a) and electroferogram (b) of double gene-site targeted tetraplex PCR for beef and buffalo authentication. In the gel image: *lane M* represents DNA ladder; *lanes 1-8* PCR products from buffalo Cytb (*lane 1*); beef ND5 (*lane 2*); beef Cytb (*lane 3*); buffalo ND5 (*lane 4*); duplex PCR of Cytb of beef and buffalo (*lane 5*); duplex PCR of ND5 of beef and buffalo (*lane 6*); triplex PCR of Cytb of beef and ND5 of beef and buffalo (*lane 7*); multiplex PCR of Cytb and ND5 of beef and buffalo (*lane 8*); and negative control (*lane* 9). The inset is the corresponding electropherogram showing all products from beef and buffalo.

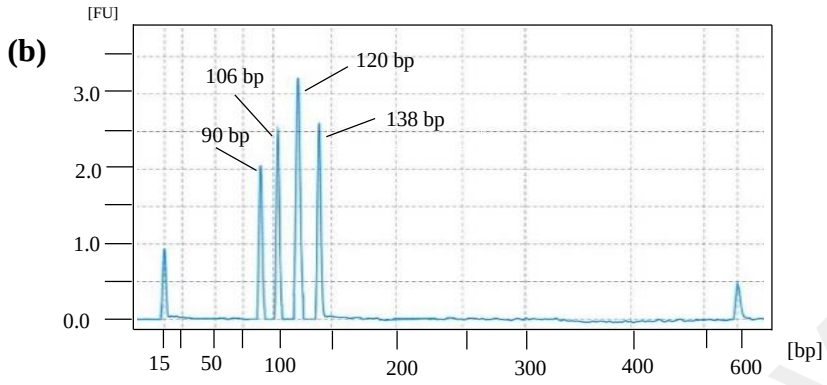


Figure 4.12: continued.

4.4.2 Tetraplex PCR Assay Specificity

The specificity of the developed tetraplex PCR assay was screened against two targets (beef and buffalo) and other 21 non-targets of terrestrial and aquatic animal species and 5 plant species (Section 3.5.2); wherein the developed tetraplex PCR system yielded PCR products only from the beef and buffalo targets and no products from non-targets (Figure 4.13).

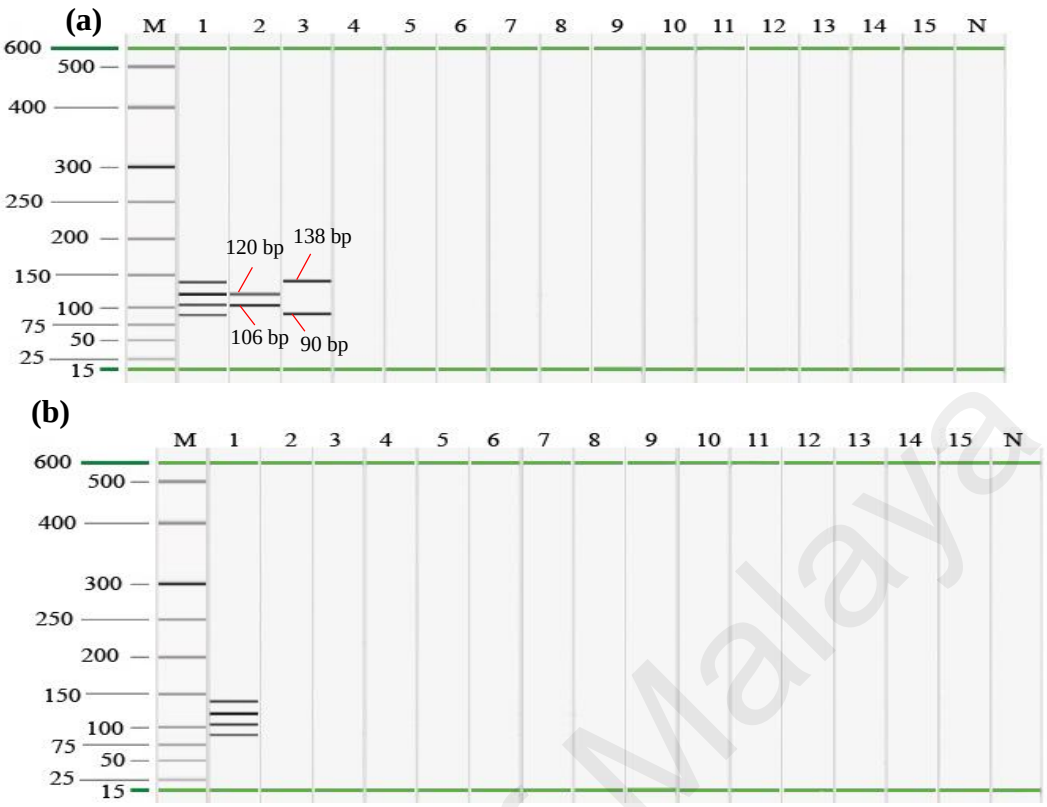


Figure 4.13: Specificity test of the developed tetraplex PCR. In the gel images of (a) and (b), M DNA ladder; N negative template control and *lane 1*, tetraplex PCR products of cytb and ND5 of beef and buffalo. In image a, *lanes 2 and 3*, PCR products of cytb and ND5 of beef and buffalo, respectively; *lanes 4-15*, PCR products from goat, lamb, dog, pork, cat, rabbit, monkey, donkey, chicken, duck, pigeon and quail respectively. In image b, *lane*s *2-15*, PCR products from rat, salmon, tuna, cod, tilapia, rohu, pangas, frog, turtle wheat, onion, garlic, ginger and pepper respectively. Corresponding electropherograms are presented by respective labels.

4.4.3 Limit of detection (LOD) of Tetraplex PCR Assay under Raw State

Extracted DNA of both target-species (cow and buffalo) was serially diluted from higher concentration (50 ng/µL) to 10.0, 5.0, 1.0, 0.5, 0.2, 0.1, 0.05, 0.02 and 0.01 ng/µL and was used as a template to determine the tetraplex PCR sensitivity since I have found spectroscopic determination of nucleic acid concentration is more reliable at higher concentration. The QIAxcel automated capillary electrophoresis produced four bands corresponding to two cytb and two ND5 genes of cow and buffalo species from as low as

0.01 ng DNA template (Figure 4.14 a). Although the band intensity of the lane 9 (0.01 ng) was very low in gel image, they were clearly reflected in the electroferograms (Lane 9 of Figure 4.14 b). Thus 0.01 ng of source DNA was defined as the limit of detection (LOD) of the developed tetraplex system.

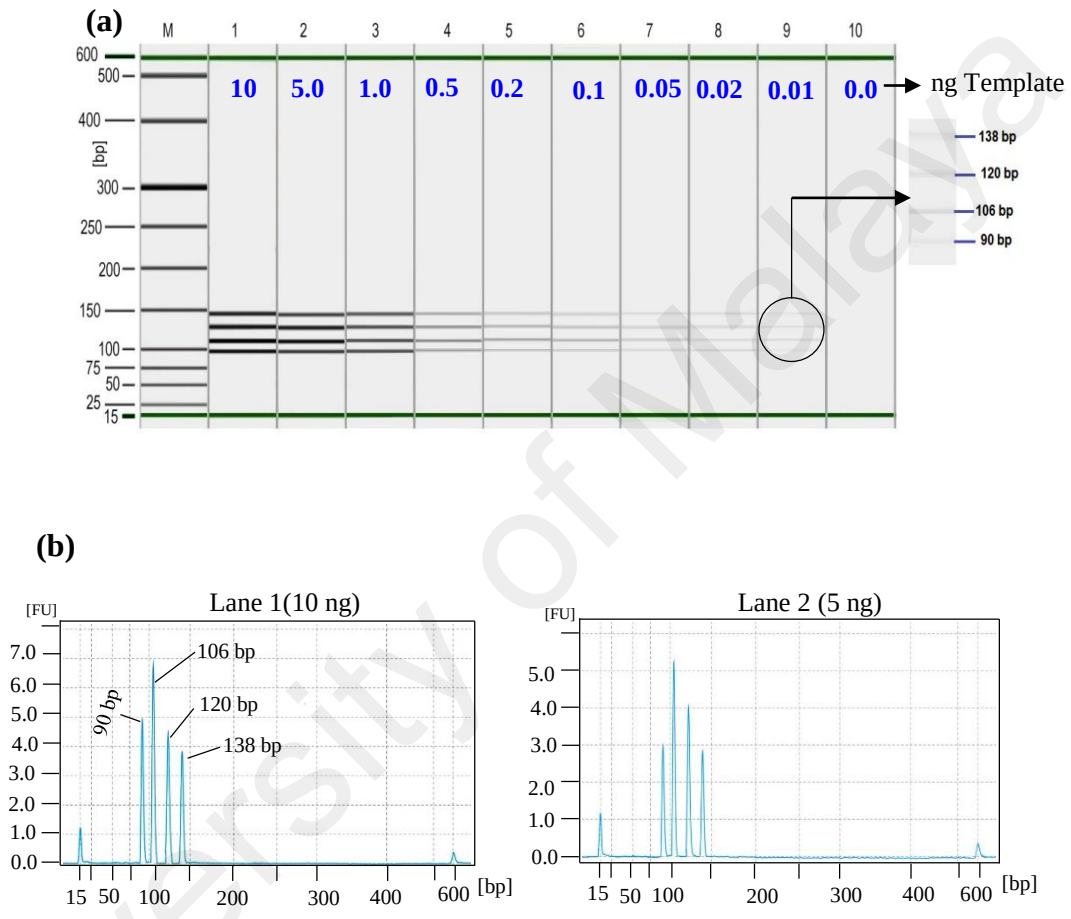


Figure 4.14: Sensitivity analysis of tetraplex PCR system. Shown are in the gel image (a), lane M is DNA ladder, lane 1–10 are the PCR products of 10, 5, 1, 0.5, 0.2, 0.1, 0.05, 0.02, 0.01 and 0 ng of DNA of cow and buffalo species and in the electropherograms (b), lanes 1–10, are presented with labels as shown in the figure.

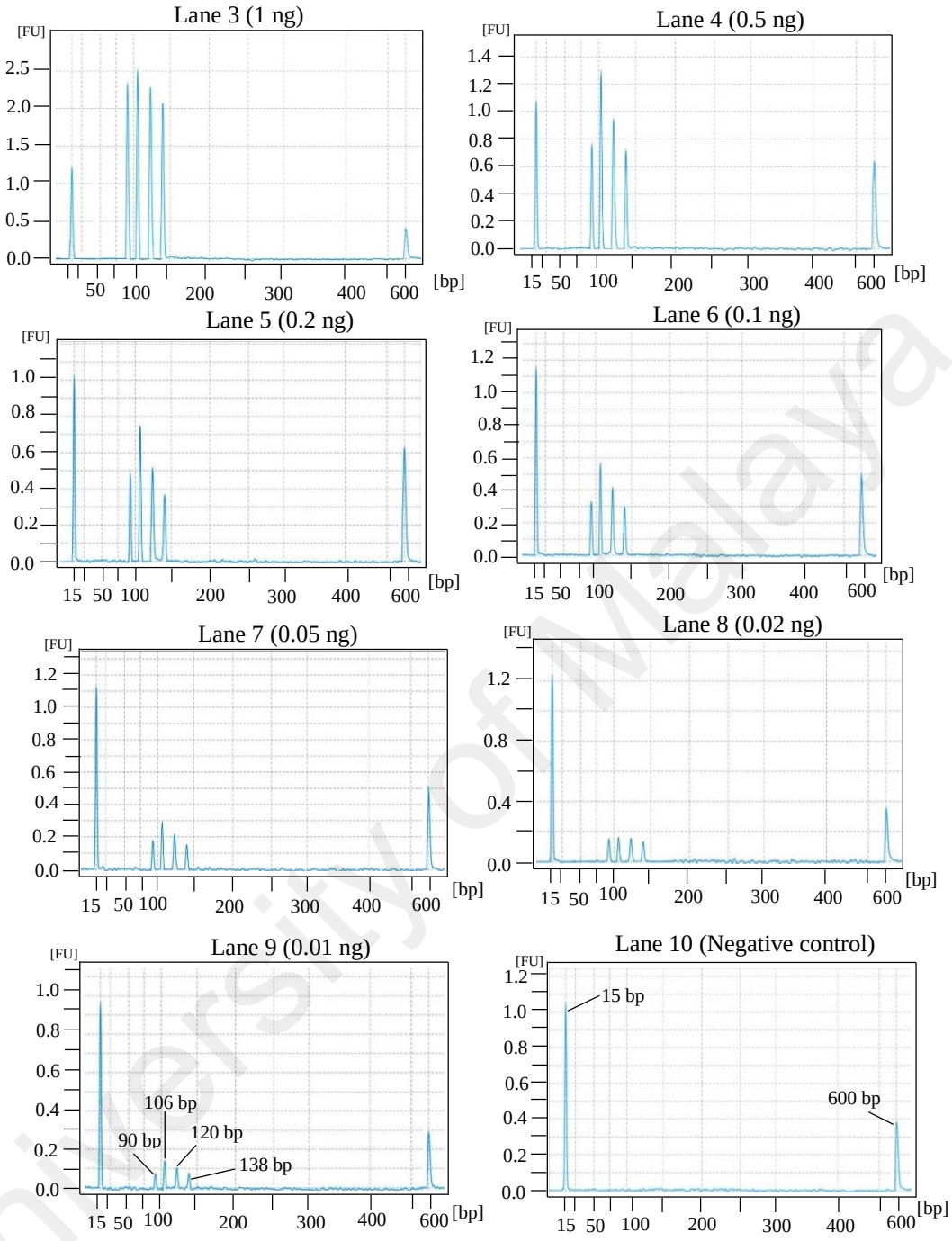


Figure 4.14: continued.

4.4.4 Sensitivity test of Tetraplex PCR Assay

4.4.4.1 Sensitivity test of Tetraplex PCR Assay under Binary Meat Admixture

To evaluate the performance of the tetraplex PCR, beef and buffalo were concomitantly detected in binary admixtures of beef and buffalo (1:99 to 99:1). The system amplified both targets (cytb and ND5) in admixtures, containing as little as 1% beef into buffalo and vice versa (Figure 4.15). While all four bands (90, 106, 120 and 138 bp) appeared from 1% to 99% beef in buffalo and vice versa, only two bands (106 and 120 bp) and (90 and 138 bp) were obtained from pure beef (lane 1) and buffalo (lane 11), respectively, clearly indicating strong specificity and sensitivity at the 1% level. Electroferograms of the image (Figure. 4.15 b) clearly revealed that when the amounts of beef DNA were decreased, the bands became fainter as might be expected and the 90 and 138 bp fragments (cytb and ND5 genes of buffalo) became more obvious, revealing correlation between the intensity and concentration.

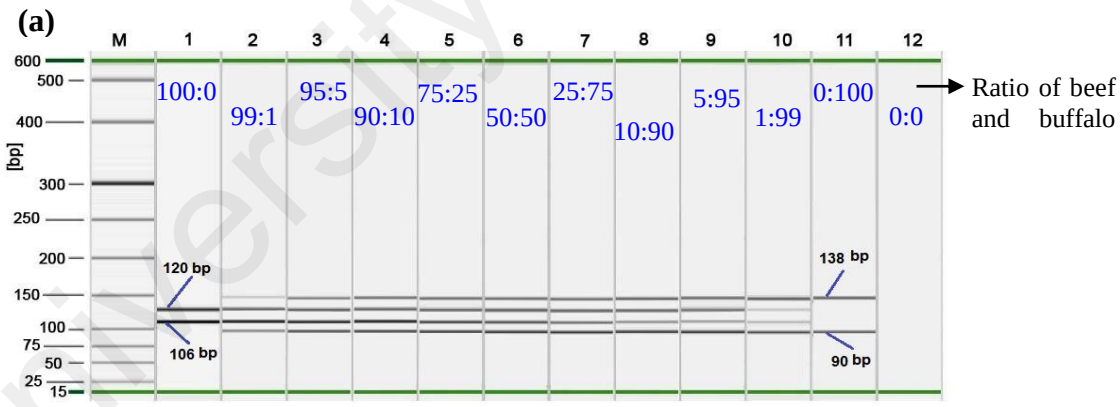


Figure 4.15: Tetraplex PCR of beef and buffalo binary ad-mixture. In the gel image (a), lane 1–11 represent PCR products of the ad-mix of beef and buffalo meat in the ratio of 100:0, 99:1, 95:5, 90:10, 75:25, 50:50, 25:75, 10:90, 5:95, 1:99 and 0:100, respectively and lane M is ladder DNA and lane 12 is negative control. The corresponding electropherograms are as shown with label (b).

(b)

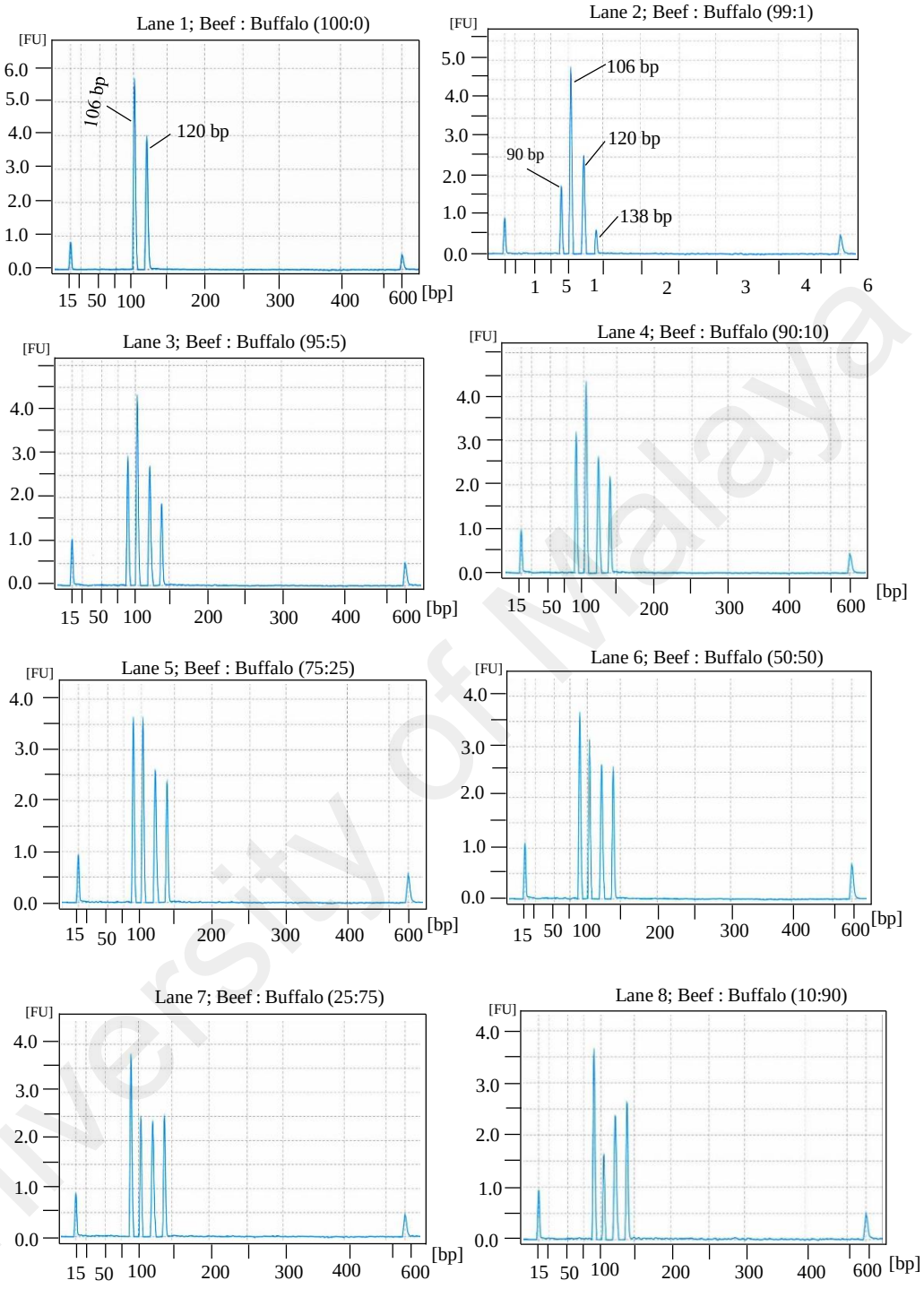


Figure 4.15: continued.

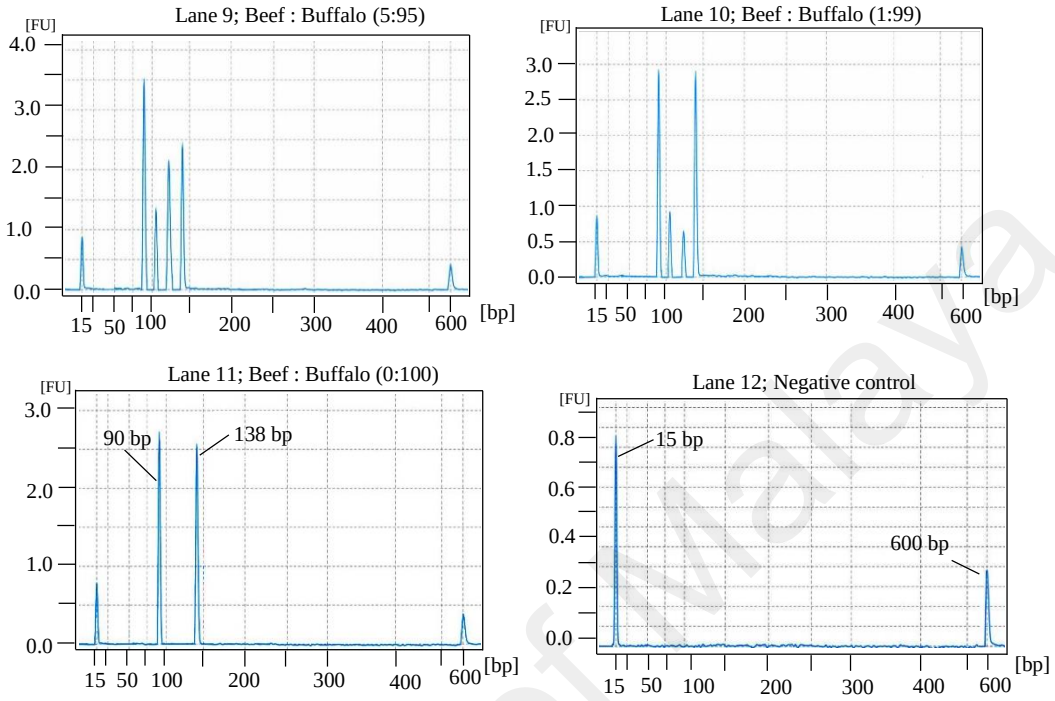


Figure 4.15: continued.

4.4.4.2 Sensitivity Test of Tetraplex PCR Assay under Commercial Product (Burger and Meat Curry)

The applicability of the tetraplex PCR assay for identifying beef and buffalo in processed foods was checked by screening seven meat curries and 45 burgers samples collected from Malaysian commercial outlets (Table 4.15).

To check the validity of the designed multiplex PCR assay, model standard, pure and deliberately adulterated (1%) beef and buffalo burgers were prepared in the laboratory as described in section 3.5.5. Experimental findings are given in Figure 4.16 and analytical data is presented in Table 4.15. Model pure beef burgers as well as commercial beef burgers amplified only beef-specific products (120 and 106 bp) (Figure 4.16; lanes 1 and 2) and pure buffalo burgers produced only buffalo-specific (90 and 138 bp) products

(Figure 4.16; lane 3). The findings were also true for 1% model beef and buffalo burgers (Figure 4.16 and lanes 4 - 9).

On the other hand, while beef was detected in all commercial beef burgers, only two of the seven beef curries were found to contain beef and five of the seven were buffalo. This reflected that fraud selling of buffalo curries in the name beef curries are rampant in Malaysia. It was noted that nobody was legally selling buffalo burgers and buffalo curries in Malaysia.

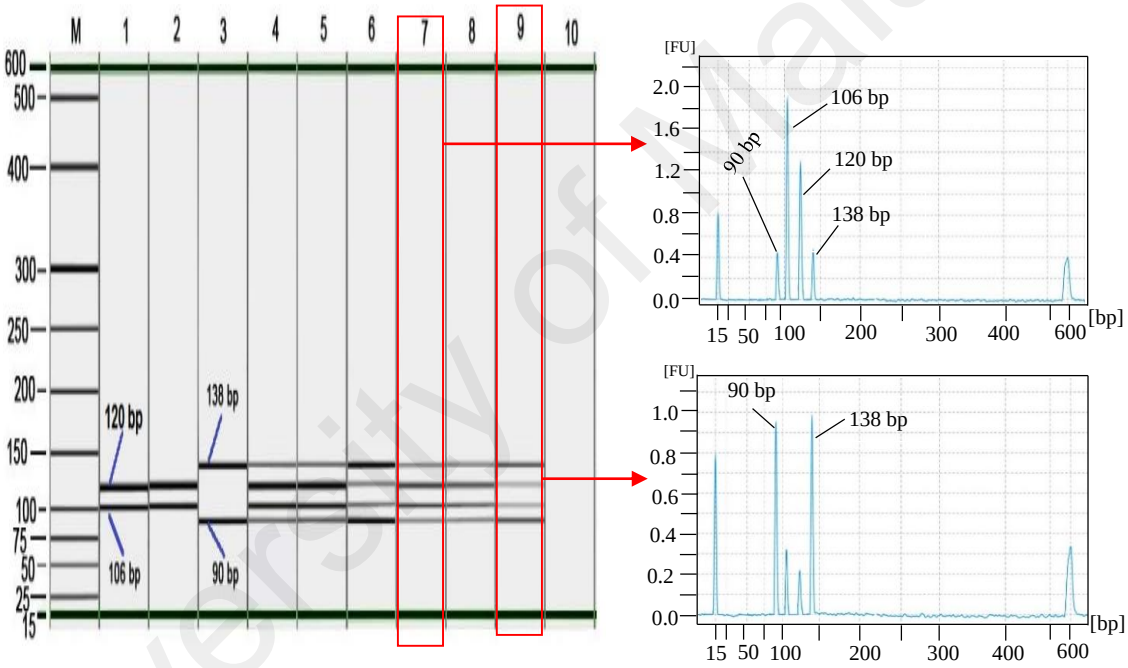


Figure 4.16: The gel image (left) and electropherograms (right-insets) of tetraplex PCR for beef *(lanes 1, 2, 4, 5, 7 & 8*) and buffalo *(lanes 3, 6 & 9)*. Shown are lab-made pure beef burger *(lanes 1)*, lab made pure buffalo burger *(Lane 3)*, lab made 1% buffalo adulterated beef burger before *(lanes 4)* and after autoclaving *(lane 7)*, lab made 1% beef adulterated buffalo burger before *(lane 6)* and after autoclaving *(lane 9*), commercial pure beef burger *(lane 2)* and 1% buffalo lab adulterated commercial beef burgers before (*lane 5*) and after autoclaving (*lane 8*). Lane M is ladder DNA and lane 10 is negative control.

Table 4.15: Analysis of model and commercial burgers under raw and processed states.

Sample	Type	Deliberately adulterated (1%)	State	Detected species		PCR accuracy (%)
				Cow	Buffalo	
Beef burger	Model	Buffalo meat	Raw	9/9	9/9	100
Beef burger	Model	Buffalo meat	Autoclaved for 2.5 h	9/9	9/9	100
Beef burger	Commercial	Buffalo meat	Raw	9/9	9/9	100
Beef burger	Commercial	Buffalo meat	Autoclaved for 2.5 h	9/9	9/9	100
Buffalo burger	Model	Beef	Raw	9/9	9/9	100
Buffalo burger	Model	Beef	Autoclaved for 2.5 h	9/9	9/9	100
Beef burger A	Commercial	-	Raw	9/9	0/9	100
Beef burger B	Commercial	-	Raw	9/9	0/9	100
Beef burger C	Commercial	-	Raw	9/9	0/9	100
Beef burger D	Commercial	-	Raw	9/9	0/9	100
Beef burger E	Commercial	-	Raw	9/9	0/9	100
Beef curry	Commercial	-	-	2/7	5/7	100

4.4.5 Target DNA Stability Test under Tetraplex PCR Assay

To evaluate the detection efficiency of the developed tetraplex PCR, various heat treated meat samples were analyzed. For this purpose, beef and buffalo meat were

subjected to three different thermal treatment processes, namely boiling, autoclaving and microwave cooking. The methods of cooking are described in earlier literatures (Ali et al., 2015d) and in section 3.5.6. The developed tetraplex PCR system successfully identified two different targets for beef and two different targets for buffalo from all thermally processed samples, including extensive autoclaving for (121^0 C at 15-psi for 2.5 h) and extensive microwaving at 700 watt for 30 min (Figure 4.17), which are known to degrade DNA.

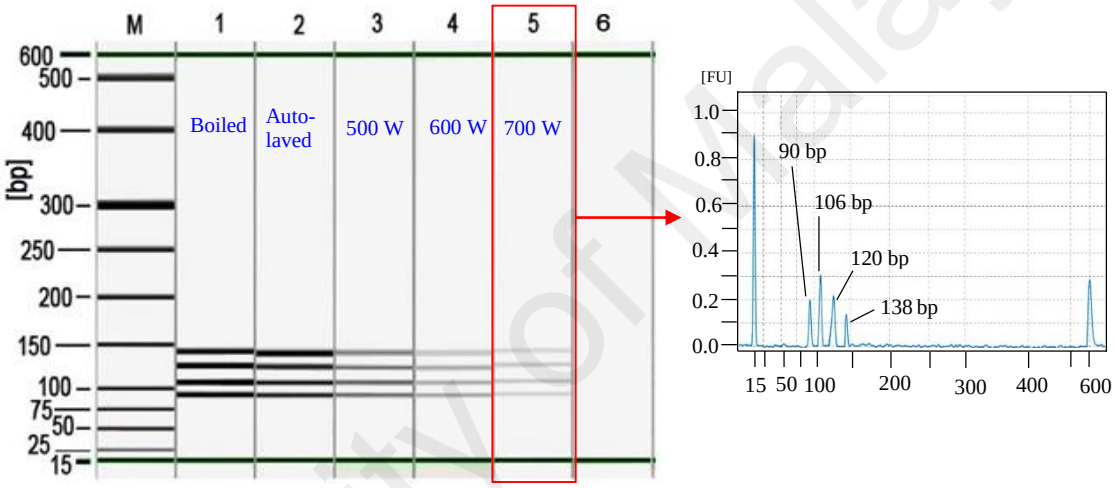


Figure 4.17: Stability test of tetratiplex PCR of DNA extracted from beef and buffalo meat *(lanes 1 - 5)* under boiling *(lane 1)*, autoclaving *(lane 2)* and microwave *(lanes 3 – 5* at 500, 600 & 700 W respectively for 30 min) cooking treatments. Lane M is ladder DNA and lane 6 is negative control.

4.5 Multiplex (Hexaplex) PCR (mPCR) Assay

4.5.1 Optimization of Multiplex (Hexaplex) PCR (mPCR) Assay

After development of tetraplex PCR assay of beef and buffalo, I included two more primer sets from cytb and ND5 genes of pig species to develop a double genes targeted multiplex (hexaplex) PCR technique for the simultaneous detection of all target species in a single assay platform. This system was developed step by step from simplex to

hexaplex as described in the section 3.5.1 and 3.6.1 (Figure 4.18). The order of the developmental stage went through the simplex (lanes 1-6), duplex (lanes 7 and 8), triplex (lane 9), tetraplex (lane 10) and hexaplex (lane 11) PCR systems to ensure the consistency of the multiplex system (Ali et al., 2015d). The simplex and all the multiplex systems (duplex, triplex, tetraplex and hexaplex) amplified the target gene (cytb and ND5) sites (120 and 106 bp for beef, 90 and 138 bp for buffalo and 146 and 73 for pig) respectively (Figure 4.18), reflecting full conformity with the simplex PCR system. Furthermore, consistent results were found in hexaplex PCR when a different multiplex PCR master mix kit (QIAGEN Multiplex PCR Plus Kit) was used.

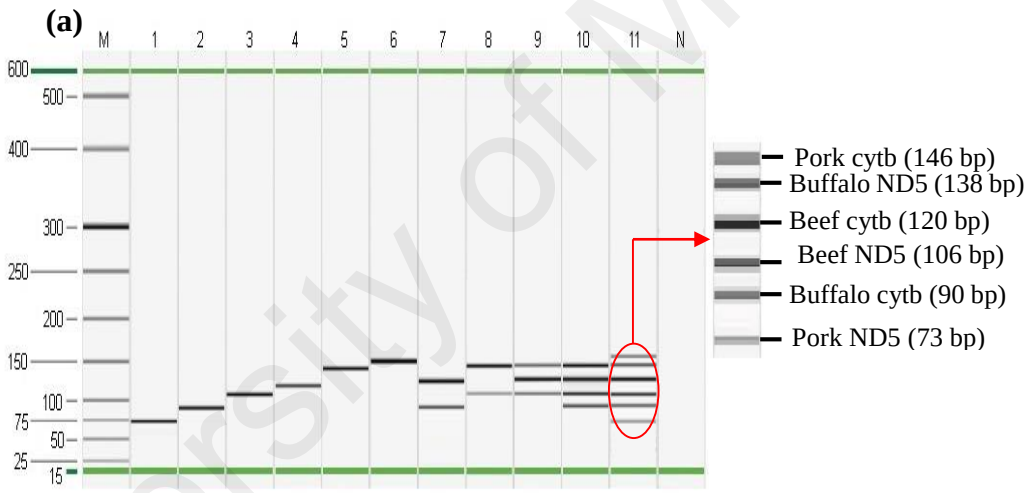


Figure 4.18: The gel image (a) and electroferogram (b) of double gene-site targeted multiplex PCR for beef, buffalo and pork authentication. In the gel image: *lane M* represents DNA ladder; *lanes 1-11* PCR products from pork ND5 (*lane 1*); buffalo cytb (*lane 2*); beef ND5 (*lane 3*); beef cytb (*lane 4*); buffalo ND5 (*lane 5*); pork cytb (*lane 6*); duplex PCR of cytb of beef and buffalo (*lane 7*); duplex PCR of ND5 of beef and buffalo (*lane 8*); triplex PCR of cytb of beef and ND5 of beef and buffalo (*lane 9*); tetraplex PCR of cytb and ND5 of beef and buffalo (*lane 10*); multiplex PCR of cytb and ND5 of beef, buffalo and pork (*lane 11*); and negative control (*lane N*). The corresponding electropherogram of *lane 11* is representated showing all products from beef, buffalo and pork.

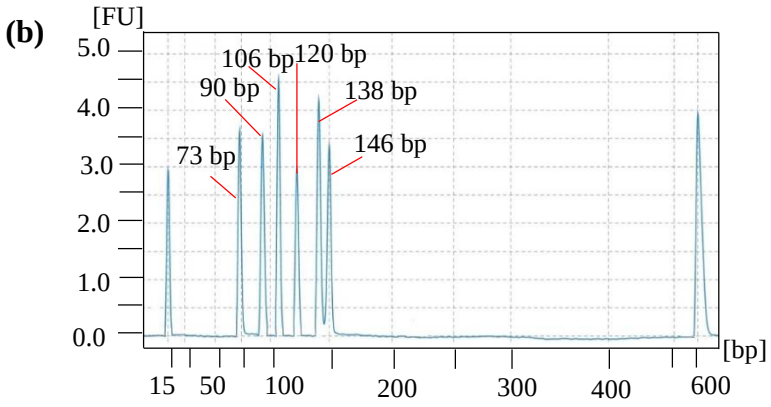


Figure 4.18: continued.

4.5.2 Multiplex PCR Assay Specificity

As like as tetraplex PCR assay, the specificity test of the developed mPCR assay was carried out against three targets (beef, buffalo and pork) and other 20 non-target of animal species and 5 plant species (Section 3.6.2) and the results revealed that the assay yielded PCR products only from the beef, buffalo and pig targets and no products from any other non-targets (Figure 4.19). The figure clearly shows that when DNA of three targets were added in a single reaction tube, three target species were amplified simultaneously (73, 90, 106, 120,138 and 146 bp products) from that tube (Figure 4.19 a, lane 1), when DNA of single target species was added, the assay amplified only the added species (in Figure 4.19 a, lane 2-4 for beef, buffalo and pork, respectively).

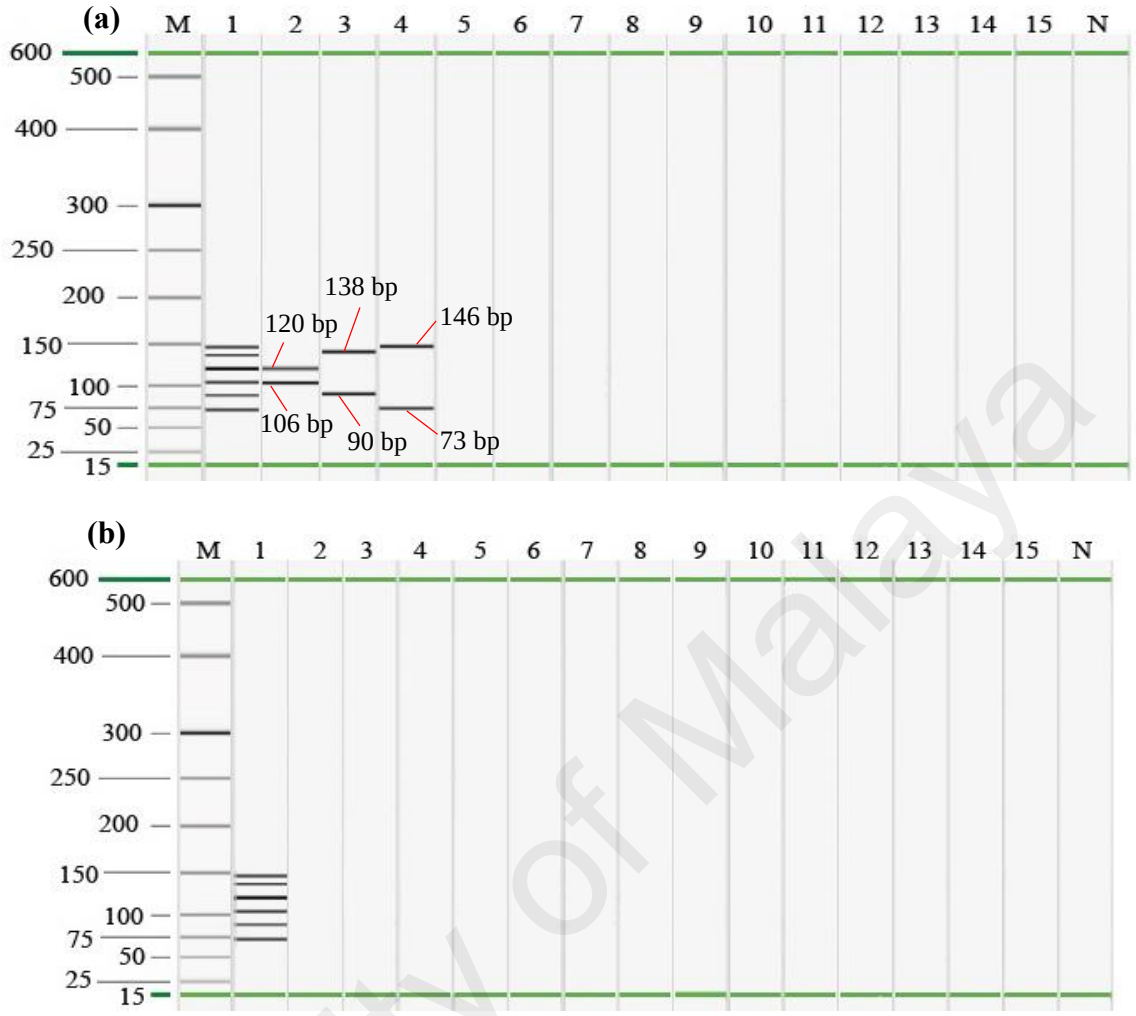


Figure 4.19: Specificity test of the developed multiplex (hexaplex) PCR (mPCR). In the gel images of (a) and (b), *lane M* DNA ladder; *lane N* negative template control and *lane 1*, mPCR products of cytb and ND5 of beef, buffalo and pork. In image a, *lanes 2, 3 and 4*, PCR products of cytb and ND5 of beef, buffalo and pork, respectively; *lanes 5-15*, PCR products from goat, lamb, dog, cat, rabbit, monkey, donkey, chicken, duck, pigeon and quail respectively. In image (b), *lane*s *2-15*, PCR products from rat, salmon, tuna, cod, tilapia, rohu, pangas, frog, turtle wheat, onion, garlic, ginger and pepper respectively.

4.5.3 Limit of Detection (LOD) of Multiplex PCR Assay under Raw State

To determine the sensitivity of the mPCR assay, extracted DNA of the target species (cow, buffalo and pig) were diluted serially from higher (50 ng/ml) to lower concentrations (10.0, 5.0,1.0, 0.5, 0.2, 0.1, 0.05, 0.02 and 0.01 ng/ml) since spectroscopic measurement at low concentration is not reproducible and trustworthy (www.biochrom.co.uk/download/72/). The QIAxcel automated capillary lectrophoresis yielded six bands corresponding to two cytb and two ND5 genes of beef, buffalo and pig species from as low as 0.02 ng of total DNA extracted from muscle tissues (Figure 4.20). The electroferogram (Figure 4.20 b) also clearly represented six peaks corresponding to the six different bands displayed in the gel- view. Thus the limit of detection (LOD) of the developed mPCR assay was concluded to be 0.02 ng DNA.

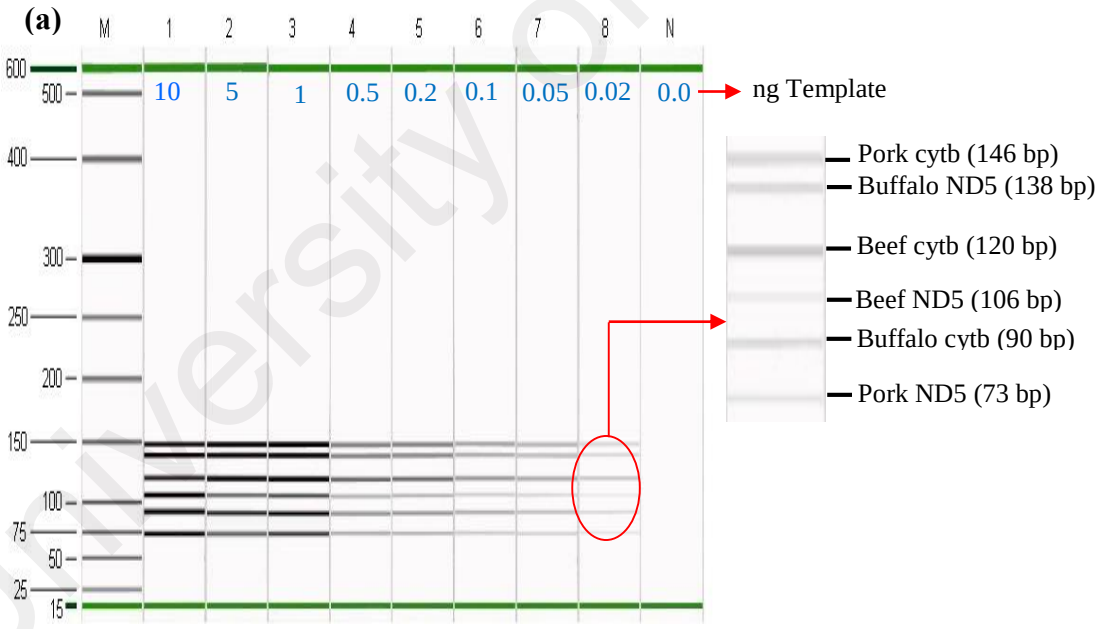


Figure 4.20: Sensitivity analysis of multiplex PCR system. Shown are in the gel view (a), lane M is DNA ladder, lane 1-8 are the PCR products of 10, 5,1, 0.5, 0.2, 0.1, 0.05 and 0.02 ng of DNA of beef, buffalo and pig species, respectively, and lane N is negative control (0 ng of DNA). The electropherogram (b) of lanes 8 is presented with labels.

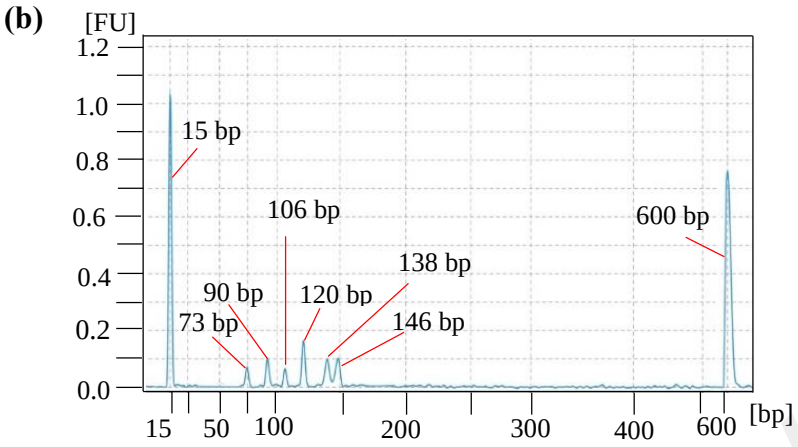


Figure 4.20: continued.

4.5.4 Sensitivity test of Multiplex PCR Assay under Commercial Meat Products (Meatballs and Frankfurters)

Meatballs and frankfurters are popular all of the world and could be consumed either as a separate menu or as additives for other dishes. Deliberately contaminated model meatballs and frankfurters of each target species were prepared in laboratory as described in section 3.6.4. The commercial meatballs of five different brands for beef (described as A-E) and pork (described as A-E) were purchased from different selling outlets across Malaysia on three different dates. Moreover, 20 beef frankfurters and 9 pork frankfurters of different brand were procured from different shops across Malaysia. The model meatballs and frankfurters of each target species were deliberately adulterated with 1%, 0.5% and 0.1% raw meat of other two target species as given in section 3.6.4. The 0.1% spiked meatballs and frankfurters of three species were autoclaved at 121^0 C for 2.5 h under 15-psi and 45-psi pressure, respectively to simulate extensive cooking effect. The experimental finding of meatballs and frankfurters are given in Figure 4.21 and Figure 4.22, respectively and the analytical data are presented in Table 4.16. The model beef, buffalo and pork meatballs and frankfurters, adulterated with 1%, 0.5% and 0.1% of

buffalo and pork; beef and pork; and beef and buffalo, amplified all the six targets (Figure 4.21; lanes 1-3; 5-7 and 9-11 and Figure 4.22; lanes 1-3; 5-7 and 9-11, respectively) representing the three target species. The 0.1% adulterated autoclaved meatballs and frankfurters also positively amplified six targets for beef, buffalo and pork (lane 4, 8 and 12 of Figure 4.21 and 4.22).

In case of commercial meatball products, only pork was contained in pork meatballs but 80% of the analyzed beef meatball was found to contain both beef and buffalo and 20% was found to contain only buffalo species. Thus the absence of pure beef meatballs in Malaysian markets reflected that buffalo substitution in beef products is rampant for the pursuit of illegal economic gain (Table 4.17). However, all the tested commercial beef frankfurters were found as both beef and buffalo positive; this indicated that all beef frankfurter products in Malaysia was buffalo adulterated. I also checked chicken and pork frankfurters, but none of them were beef and buffalo positive; this was probably because the prices of beef and buffalo are higher than those of chicken and pork (Table 4.17).

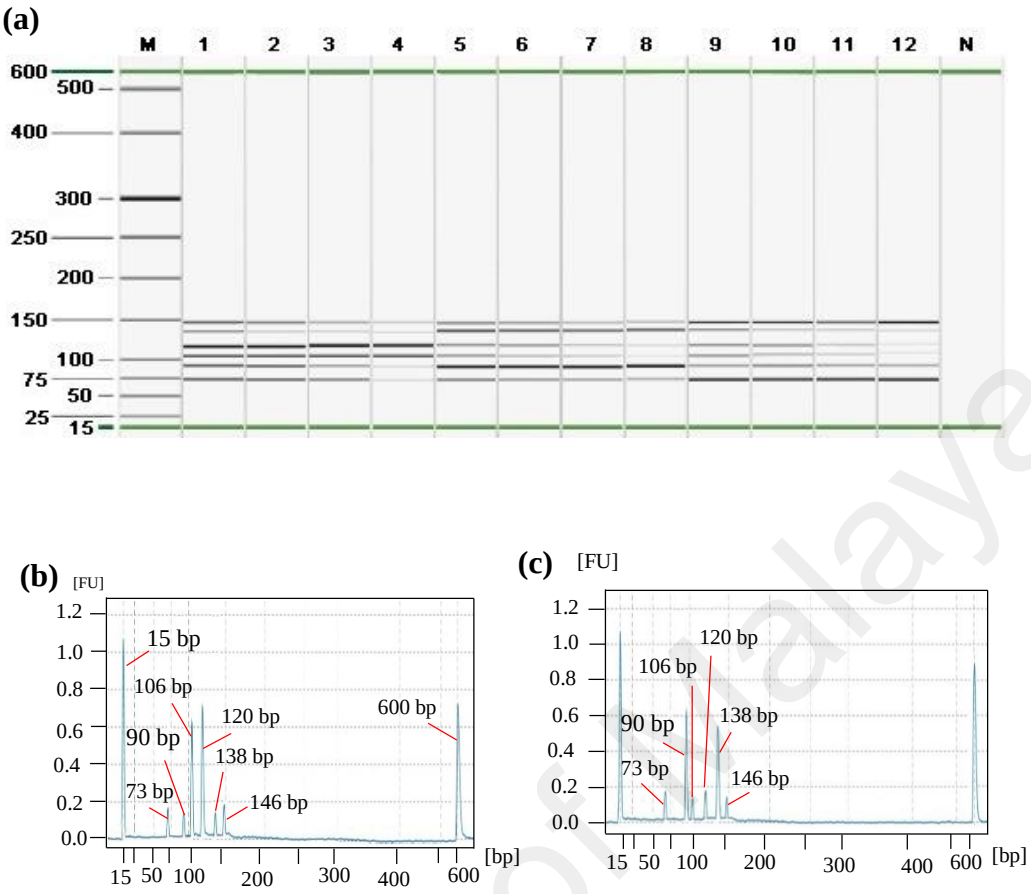


Figure 4.21: The gel image (a) and electropherograms (b-d) of multiplex PCR (mPCR) for the detection of double gene-targeting cow, buffalo and pig in deliberately adulterated model beef, buffalo and pork meatball under raw and processed states. In the gel image; lane M, Ladder; lanes 1-3, m-PCR of beef meatball spiked with 1%, 0.5% and 0.1% of buffalo and pork, respectively, under raw state; lanes 5-7, mPCR of buffalo meatball spiked with 1%, 0.5% and 0.1% of beef and pork, respectively, under raw state; lanes 9-11, mPCR of pork meatball spiked with 1%, 0.5% and 0.1% of beef and buffalo, respectively, under raw state; lane 4, 8 and 12, mPCR of heat treated (autoclaved for 2.5 h) 0.1% adulterated beef, buffalo and pork meatballs respectively; lane N negative control. The corresponding electroferograms of lane 4, 8 and 12 are shown with labels in b, c and d, respectively.

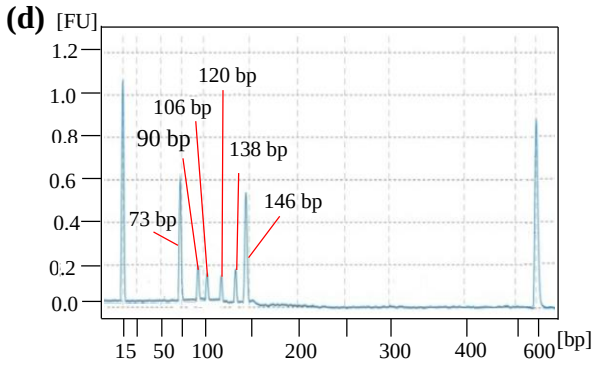


Figure 4.21: continued.

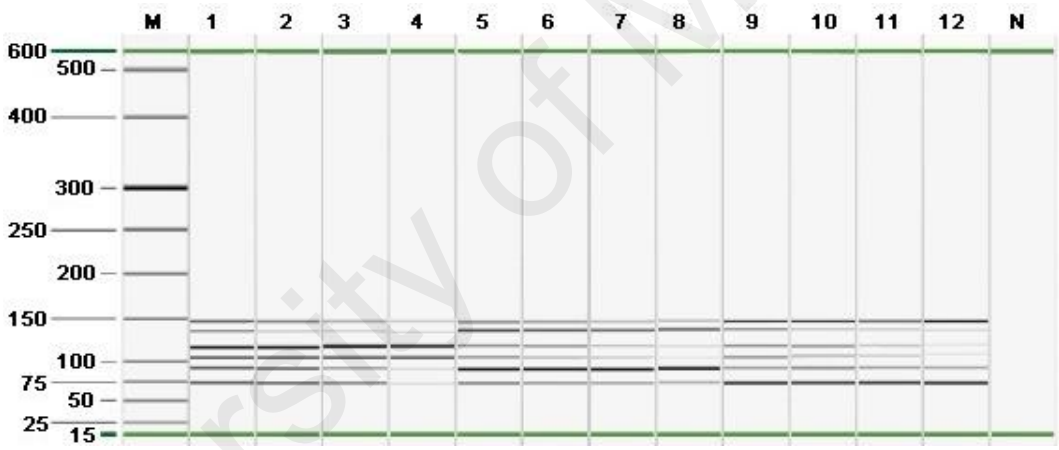


Figure 4.22: Gel image (a) and the electropherograms (b−d) of mPCR for the detection of double gene-targeted beef, buffalo, and pork in deliberately adulterated model beef, buffalo, and pork frankfurters under raw and processed states. In the gel image, M, Ladder; lanes 1−3, m-PCR of beef frankfurter spiked with 1%, 0.5%, and 0.1% of buffalo and pork, respectively, under raw state; lanes 5−7, mPCR of buffalo frankfurter spiked with 1%, 0.5%, and 0.1% of beef and pork, respectively, under raw state; lanes 9−11, mPCR of pork frankfurter spiked with 1%, 0.5%, and 0.1% of beef and buffalo, respectively, under raw state; lanes 4, 8, and 12, mPCR of heat-treated (autoclaved for 2.5 h) 0.1% adulterated beef, buffalo, and pork frankfurter, respectively; lane N, negative control. The corresponding electroferograms of lane 4, 8, and 12 are shown labeled as b, c, and d, respectively.

Table 4.16: Screening of model meatball and frankfurter products using developed multiplex PCR.

Sample	Adulteration		State	Detected species			PCR accuracy (%)
	species	%		Beef	Buffalo	Pork	
Model meatballs							
Beef meatball	Buffalo and pork	1.0	Raw	9/9	9/9	9/9	100
Beef meatball	Buffalo and pork	0.5	Raw	9/9	9/9	9/9	100
Beef meatball	Buffalo and pork	0.1	Raw	9/9	9/9	9/9	100
Beef meatball	Buffalo and pork	0.1	Autoclaved for 2.5 h	9/9	9/9	9/9	100
Buffalo meatball	Beef and pork	1.0	Raw	9/9	9/9	9/9	100
Buffalo meatball	Beef and pork	0.5	Raw	9/9	9/9	9/9	100
Buffalo meatball	Beef and pork	0.1	Raw	9/9	9/9	9/9	100
Buffalo meatball	Beef and pork	0.1	Autoclaved for 2.5 h	9/9	9/9	9/9	100
Pork meatball	Beef and Buffalo	1.0	Raw	9/9	9/9	9/9	100
Pork meatball	Beef and Buffalo	0.5	Raw	9/9	9/9	9/9	100
Pork meatball	Beef and Buffalo	0.1	Raw	9/9	9/9	9/9	100
Pork meatball	Beef and Buffalo	0.1	Autoclaved for 2.5 h	9/9	9/9	9/9	100

Note: The numerator and denominator of each fraction denote the number of positive detection and total number of samples analysed using the multiplex PCR assay.

Table 4.16: continued.

Sample	Adulteration		State	Detected species			PCR accuracy (%)
	species	%		Beef	Buffalo	Pork	
Model frankfurters							
Beef frankfurter	Buffalo and Pork	1.0	Raw	9/9	9/9	9/9	100
Beef frankfurter	Buffalo and Pork	0.5	Raw	9/9	9/9	9/9	100
Beef frankfurter	Buffalo and Pork	0.1	Raw	9/9	9/9	9/9	100
Beef frankfurter	Buffalo and Pork	0.1	Autoclaved for 2.5 h	9/9	9/9	9/9	100
Buffalo frankfurter	Beef and Pork	1.0	Raw	9/9	9/9	9/9	100
Buffalo frankfurter	Beef and Pork	0.5	Raw	9/9	9/9	9/9	100
Buffalo frankfurter	Beef and Pork	0.1	Raw	9/9	9/9	9/9	100
Buffalo frankfurter	Beef and Pork	0.1	Autoclaved for 2.5 h	9/9	9/9	9/9	100
Pork frankfurter	Beef and Buffalo	1.0	Raw	9/9	9/9	9/9	100
Pork frankfurter	Beef and Buffalo	0.5	Raw	9/9	9/9	9/9	100
Pork frankfurter	Beef and Buffalo	0.1	Raw	9/9	9/9	9/9	100
Pork frankfurter	Beef and Buffalo	0.1	Autoclaved for 2.5 h	9/9	9/9	9/9	100

Table 4.17: Screening of Commercial meatball, frankfurter and beef curry samples using developed multiplex PCR.

Sample	State	Detected species			PCR accuracy (%)
		Beef	Buffalo	Pork	
Commercial meatballs					
Beef meatball A	Raw	9/9	9/9	0/9	100
Beef meatball B	Raw	9/9	9/9	0/9	100
Beef meatball C	Raw	6/9	9/9	0/9	100
Beef meatball D	Raw	7/9	9/9	0/9	100
Beef meatball E	Raw	5/9	9/9	0/9	100
Pork meatball A	Raw	0/9	0/9	9/9	100
Pork meatball B	Raw	0/9	0/9	9/9	100
Pork meatball C	Raw	0/9	0/9	9/9	100
Pork meatball D	Raw	0/9	0/9	9/9	100
Pork meatball E	Raw	0/9	0/9	9/9	100
Commercial frankfurters					
Beef frankfurter	Raw	20/20	20/20	0/20	100
Chicken frankfurter	Raw	0/10	0/10	0/10	100
Pork frankfurter	Raw	0/10	0/10	10/10	100

Note: The numerator and denominator of each fraction denote the number of positive detection and total number of samples analysed using the multiplex PCR assay.

4.5.5 Target DNA Stability Test under Multiplex PCR Assay

To assess the detection efficiency of the described mPCR assay, various thermally treated meat samples were analysed. As reported in methodology (section 3.6.5), raw meat of beef, buffalo and pork were subjected to three different heat treatment processes, namely boiling, autoclaving and microwave cooking. The extracted DNA of the all processed meat samples were used as templates for the mPCR assay. The system successfully detected all target species, beef, buffalo and pork under all thermal

processing conditions, including extensive autoclaving (121^{0}C at 15-psi for 2.5 h) and extreme microwaving at 700W for 30 min (Figure 4.23).

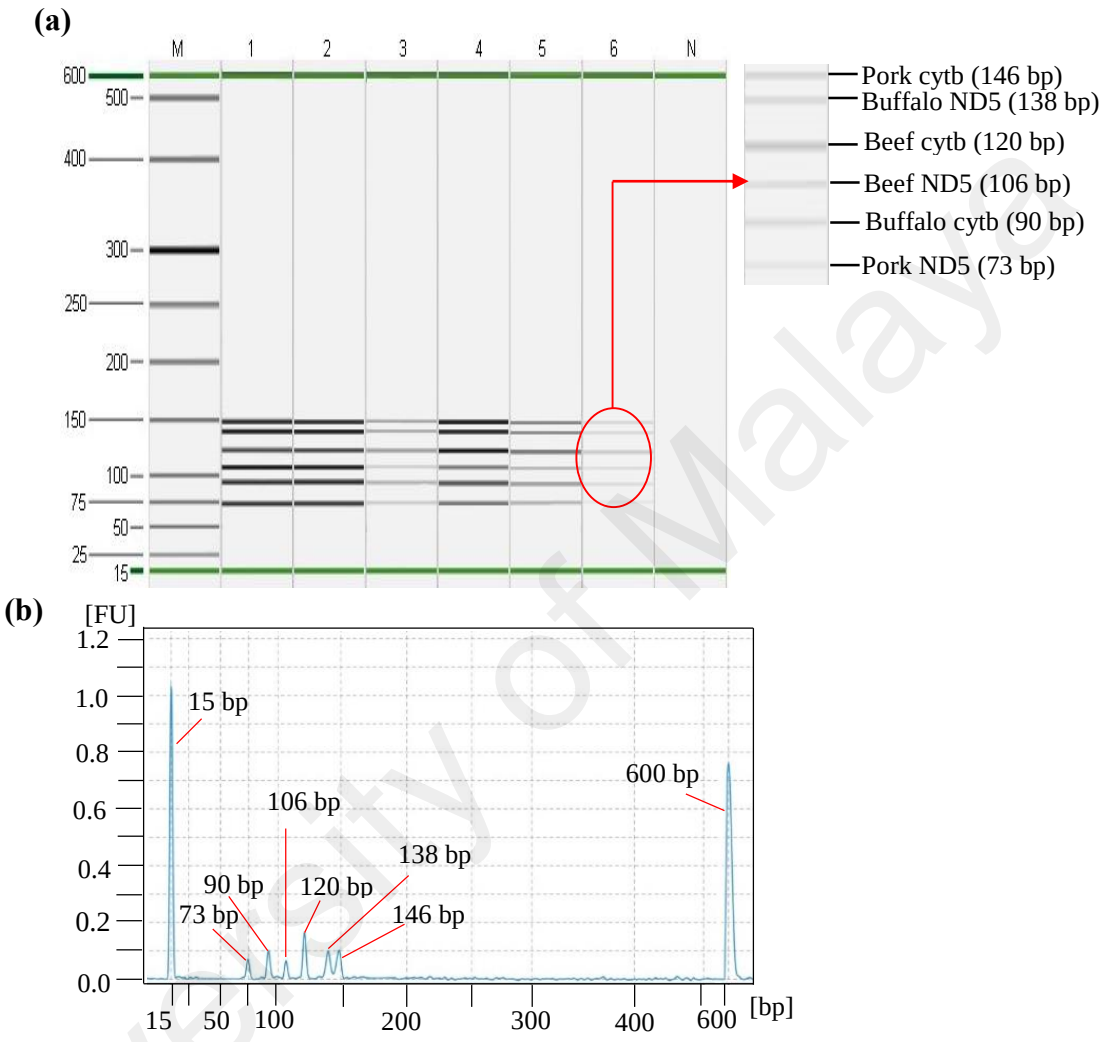


Figure 4.23: The gel image (a) and electroferogram (b) of the stability test of multiplex PCR of DNA extracted from beef, buffalo and pork (lanes 1-6) under boiling (lane 1), autoclaving (lane 2 and 3 for 20 min and 2.5 h respectively) and microwaving (lanes 4-6 at 500, 600 & 700Wrespectively for 30 min) cooking treatments. LaneMis ladder DNA and lane N is negative control. The corresponding electroferogram (b) of lane 6 is shown with labels.

4.6 PCR Products Authentication by RFLP Analysis

4.6.1 RFLP Analysis of Beef and Buffalo PCR Products

4.6.1.1 Authentication of Beef and Buffalo PCR Products of raw meat by RFLP Analysis

In this study, the tetraplex PCR products of beef and buffalo were digested simultaneously with three restriction enzymes as cited in section 3.7.1.1 and 3.7.1.2, and clear fingerprints were obtained for each of the four different targets (Figure 4.24 and Table 3.9). First, each target was digested separately with an appropriate RE (Table 3.9) to study its individual restriction patterns (Figure 4.24). Both buffalo cytb (90 bp) (Figure 4.24, lane 1) and beef ND5 (106 bp) (Figure 4.24, lane 3) products were digested by FatI RE, which generated two fragments for each target (50 and 40 bp for buffalo cytb (lane 2) and 87 and 19 bp for beef ND5 (lane 4)). On the other hand, beef cytb (120 bp) (lane 5) was digested by EciI that produced two fragments (75 and 45 bp) (lane 6). In contrast, buffalo ND5 product (lane 7) was digested with AluI, which resulted in another two fragments (130 and 8 bp) (lane 8). However, 8 bp fragment was not detected because it went beyond the lower limit of instrumental resolution, which was ≤15 bp. Finally, the tetraplex PCR products (lane 9) were subjected to RE digestion with the three enzymes (FatI, EciI, and AluI) in a single tube, and this generated molecular fingerprints which were composed of a total of seven fragments (19, 40, 45, 50, 75, 87, and 130) (lane 10). The origins of these products (lane 9) were confirmed by the separate digests of the four targets (lanes 1−8).

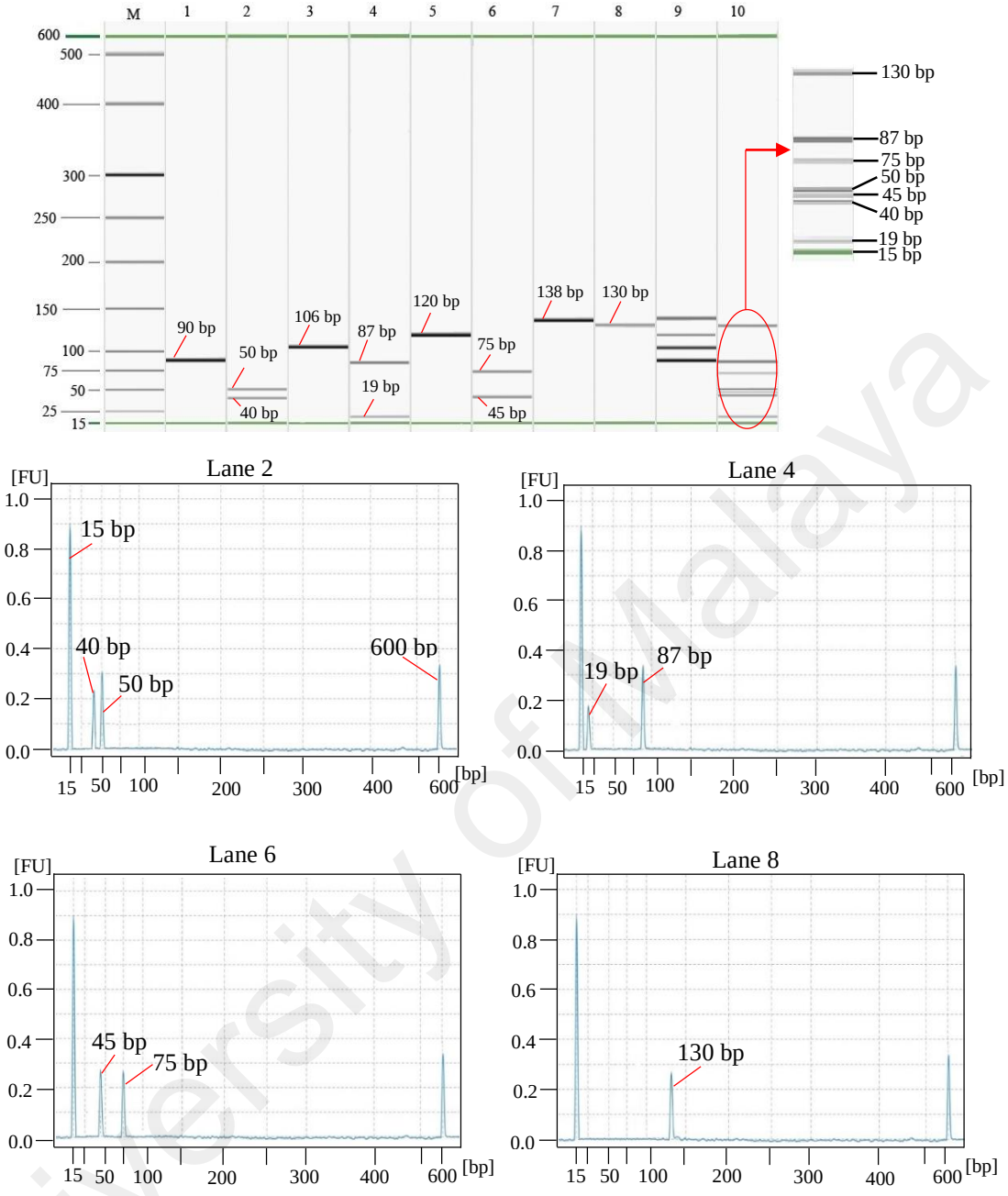


Figure 4.24: RFLP analysis of simplex and mPCR products before (lanes 1, 3, 5, 7, and 9) and after (lanes 2, 4, 6, 8, and 10) restriction digestion. In the gel image, lanes 1 and 2, cytb of buffalo; lanes 3 and 4, ND5 of beef; lanes 5 and 6, cytb of beef; lanes 7 and 8, ND5 of buffalo; and lanes 9 and 10, mPCR of cytb and ND5 of beef and buffalo. Corresponding electropherograms are shown with labels.

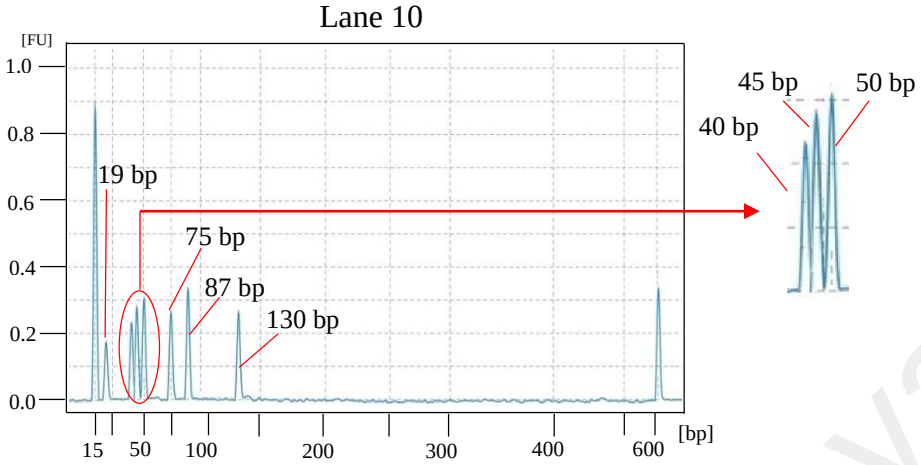


Figure 4.24: continued.

4.6.1.2 Authentication of Tetraplex PCR Products of frankfurters by RFLP Analysis

After the tetraplex PCR-RFLP assay under pure states was optimized, it was subsequently optimized and evaluated for the screening of commercial beef and buffalo frankfurters under raw, boiled, and autoclaved states (Taboada et al., 2014). Dummy frankfurters were deliberately adulterated, and their restriction digestion patterns were studied (Figure 4.25). The digest of all samples (lanes 1, 3, 5, 7, 9, and 11) clearly presented the signature fingerprints of 7 fragments (lanes 2, 4, 6, 8, 10, and 12), the stability of the four biomarkers were not affected by several thermal treatment.

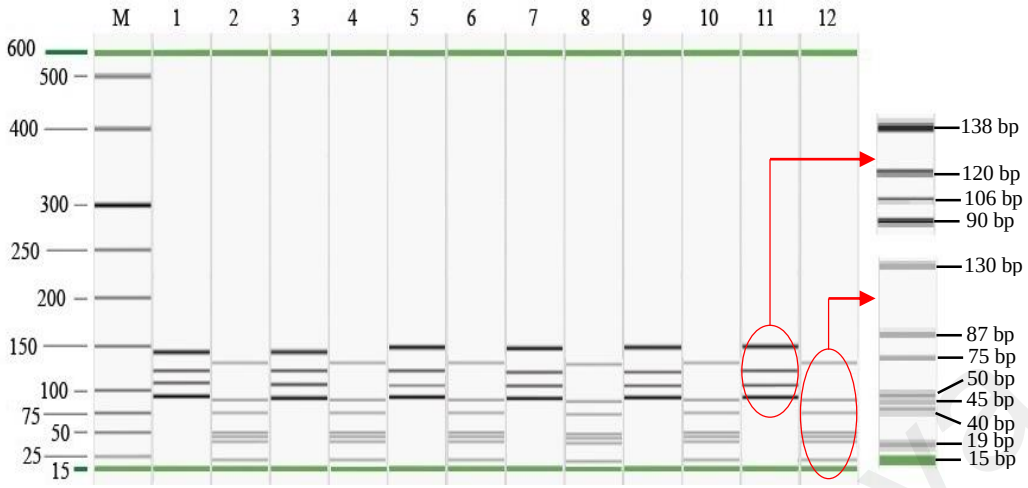


Figure 4.25: PCR-RFLP analysis of mPCR products of deliberately adulterated raw and heat-treated (boiled and autoclaved) beef (lanes 1−6) and buffalo (lanes 7−12) frankfurters. In gel image, lanes 1 and 2,buffalo-adulterated raw beef frankfurter before and after digestion, respectively; lanes 3 and 4,buffalo-adulterated boiled (98 °C for 90 min) beef frankfurter before and after digestion, respectively; lanes 5 and 6,buffalo-adulterated autoclaved (121 °C and 15 psi pressure for 2.5 h) beef frankfurter before and after digestion, respectively; lanes 7 and 8, beef-adulterated raw buffalo frankfurter before and after digestion, respectively; lanes 9 and 10, beef-adulterated boiled (98 °C for 90 min) buffalo frankfurter before and after digestion, respectively; lanes 11 and 12, beef-adulterated autoclaved (121 °C and 15 psi pressure for 2.5 h) buffalo frankfurter before and after digestion, respectively.

4.6.2 Authentication of Pork PCR Products of Raw Meat and Frankfurter by RFLP Analysis

Simplex PCR products of pork Pocytb and PoND5 were digested individually with CviKI-1 and FatI RE, respectively, because in silico studies demonstrated overlapping fragments with beef and buffalo. Post digested PoND5 PCR product (73 bp) (Figure 4.26, lane 1) produced 2 fragments of 52 and 21 bp (Figure 4.26, lane 2), and Pocytb PCR product (146 bp) (lane 3) generated 3 fragments of 80, 45, and 21 bp (lane 4). Similar products were found from boiled (98 °C for 90 min) and autoclaved (121 °C at 45 psi for

2.5 h) pork frankfurters. The restriction digestion maps of different heat-treated (boiled and autoclaved) samples were similar to those from the raw sample (Figure 4.27).

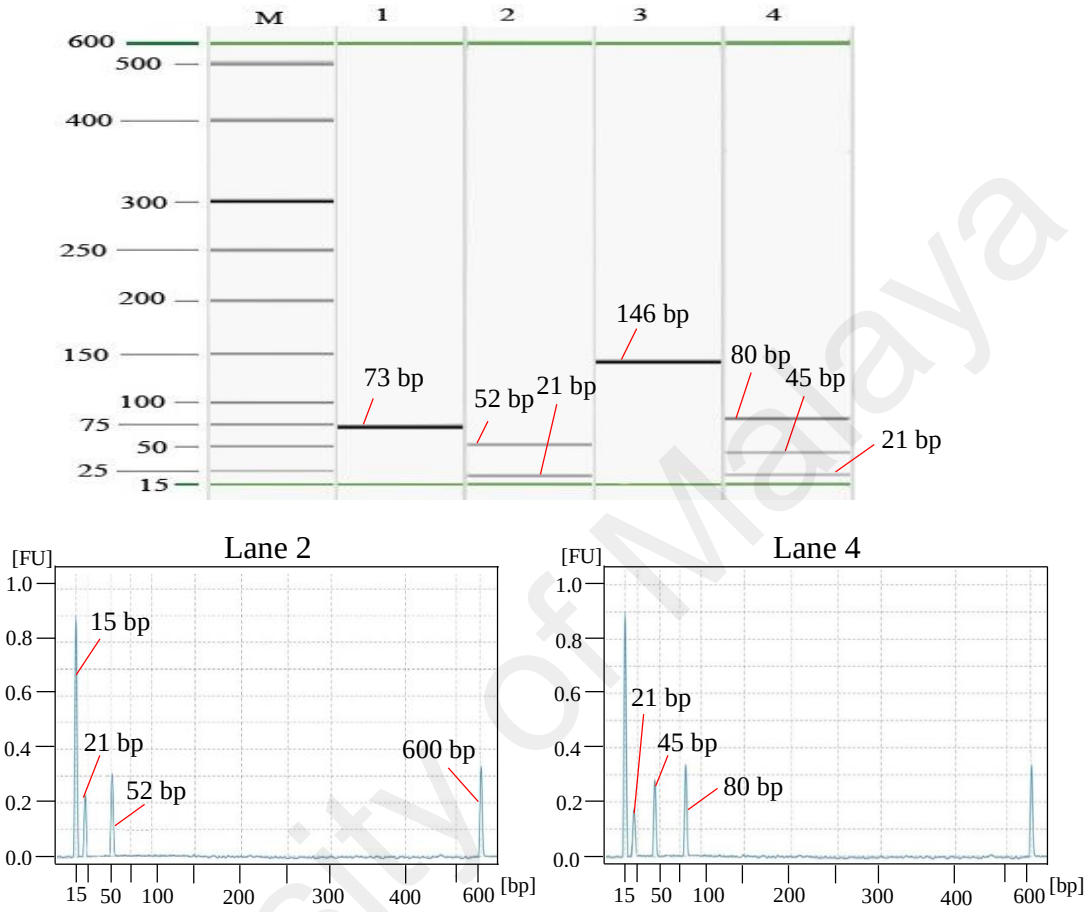


Figure 4.26: PCR-RFLP analysis of simplex PCR products of pork PoND5 and Pocytb before and after restriction endonuclease digestion. In the gel image, lanes 1 and 2, PCR products of PoND5 before and after digestion; lanes 3 and 4, products of Pocytb before and after digestion, respectively. Corresponding electropherograms are indicated by corresponding labels.

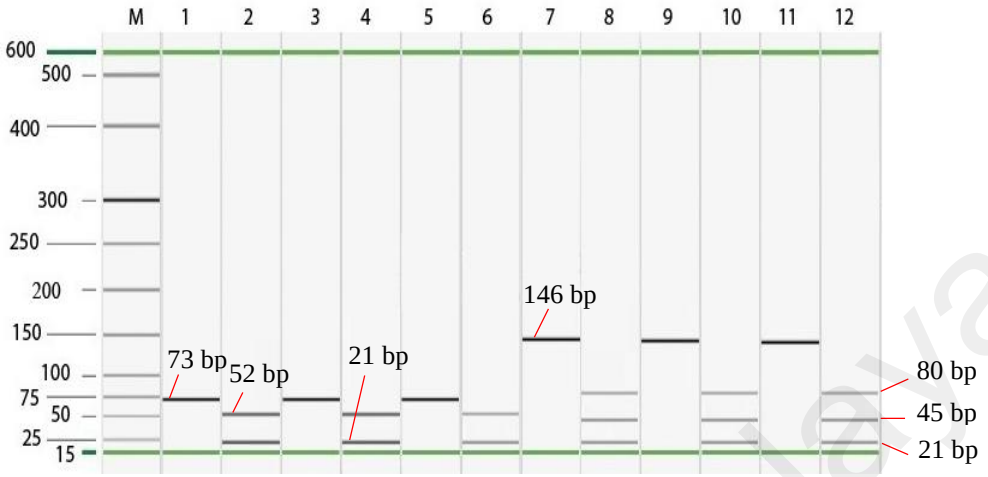


Figure 4.27: RFLP analysis of pork PoND5 (lanes 1−6) and Pocytb (lanes 7−12) PCR products before (lanes 1, 3, 5, 7, 9, and 11) and after (lanes 2, 4, 6, 8, 10, and 12) restriction digestion. In the gel view, PCR products from raw (lanes 1, 2, 7, and 8), boiled (lanes 3, 4, 9, and 10), and autoclaved (lanes 5, 6, 11, and 12) pork frankfurter; lane M, DNA ladder.

4.7 Real-time PCR Assay

4.7.1 Multiplex Real-time PCR System

A singleplex qPCR system for each individual species was optimized one by one using the respective primers and probes for each of the three target species and after that additional species (primers and probes) were added sequen- tially one after another into the reaction mixture to optimize the final tetraplex qPCR system. The Ct values of tetraplex qPCR assay were Ct = 18.74 ± 0.04, 17.75 ± 0.06, 14.80 ± 0.05, and 15.14 ± 0.05 that nicely matched with the qPCR Ct for cow, buffalo, pig, and IAC, respectively, effectively confirming that there were not any significant variation of Ct values when the platforms were changed from single to multiplex (Figure 4.28).

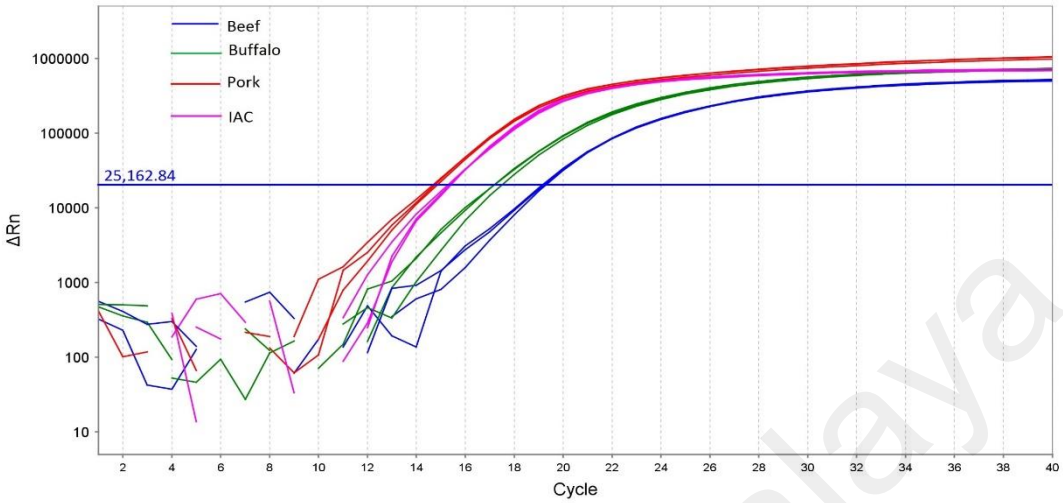


Figure 4.28: Amplification plot of tetraplex qPCR for cow, buffalo, pig and endogenous control. Color of curve indicates specific species; blue for cow, green for buffalo, red for pig and pink for endogenous control.

4.7.2 Specificity of the Multiplex Real-time PCR System

The nucleic acid sequences of the designed primer sets and probes were screened with NCBI database using online Basic Local Alignment Search Tool (BLAST) and the results were found to have completely identical sequences with target species and sufficient mismatch with other species. On the other hand, alignment of both primer sets and probes sequences with 29 target and non-target species using MEGA5 software showed 100% sequence similarity with target species and multiple nucleotide mismatches (3-18 nt in primers and 3-25 nt in probes) with other related or non-target species (Table 4.2-4.7), indicating that there were no or very little chances for amplifying a cross-species.

The experimental specificity of the tetraplex qPCR system was evaluated with 30 ng of DNA extracted from fresh muscle tissues of three target species (cattle, buffalo, and pig) and 25 nontarget species (lamb, goat, cat, dog, pigeon, chicken, quail, duck, rat,

monkey, rabbit, donkey, tilapia, tuna, rohu, salmon, cod, pangas, turtle, frog, onion, ginger, wheat, garlic, and pepper) on three different days in triplicate. The amplification profile clearly demonstrated the species specific amplification curves as well as background fluorescence for the relevant species in a 40 cycle PCR assay, confirming the absence of any cross-amplifications (Figure 4.29). Additionally, the IAC that amplified eukaryotic target from all species reflected that good quality DNA template was present in all tubes (Figure 4.29). While the amplification signals (Ct values) of the tetraplex qPCR assay for the target species were 18.84 ± 0.06, 17.86 ± 0.03, and 14.83 ± 0.08 for cattle, buffalo, and pig, respectively, the non-target species did not yield any detectable Ct during the 40 cycle PCR reaction (Table 4.18). On the other hand, Ct values of IAC for all the target and nontarget species were 15.61−18.50 (Table 4.18), eliminating the chances of any false positive detection.

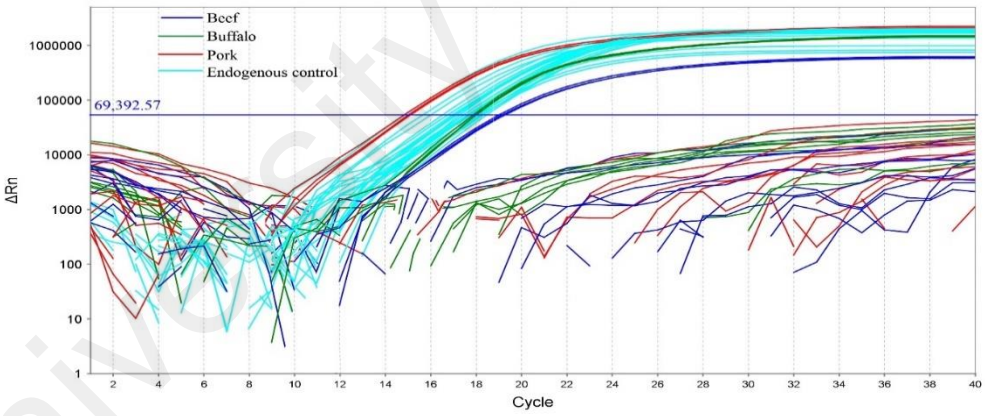


Figure 4.29: Multiplex qPCR amplification plot for porcine (red), cattle (blue), and buffalo (green) species along with the endogenous control for eukaryotes (sky blue) against 25 species (below the threshold cycle).

Table 4.18: Specificity/cross-reactivity tests of multiplex qPCR and endogenous system.

Animal species tested	Multiplex real-time PCR system		Endogenous PCR system	
	Increase of fluorescence signal	Mean Ct value	Increase of fluorescence signal	Mean Ct value
Cow	+	18.84±0.06	+	16.53±0.13
Buffalo	+	17.86±0.03	+	15.80±0.15
Pig	+	14.83±0.08	+	16.33±0.07
Sheep	-	-	+	17.30±0.04
Goat	-	-	+	17.51±0.13
Cat	-	-	+	18.06±0.10
Dog	-	-	+	17.82±0.08
Pigeon	-	-	+	15.92±0.11
Chicken	-	-	+	17.50±0.11
Quail	-	-	+	17.81±0.06
Duck	-	-	+	18.43±0.09
Rat	-	-	+	16.94±0.08
Monkey	-	-	+	15.66±0.13
Rabbit	-	-	+	15.78±0.08
Donkey	-	-	+	18.07±0.04
Tilapia	-	-	+	17.45±0.13
Tuna	-	-	+	17.16±0.10
Rohu	-	-	+	16.59±0.12
Salmon	-	-	+	16.43±0.09
Cod	-	-	+	17.61±0.06
Panga	-	-	+	18.46±0.13
Turtle	-	-	+	16.69±0.15
Frog	-	-	+	17.72±0.11
Onion	-	-	+	18.07±0.14
Ginger	-	-	+	16.03±0.05
Wheat	-	-	+	17.33±0.08
Garlic	-	-	+	17.75±0.14
Pepper	-	-	+	15.37±0.12

Note: '+' Positive PCR result (Ct value < 40) and '-' no increase of the fluorescence signal within 40 cycles.

4.7.3 Limit of Detection (LOD)

In this assay, the LOD of the tetraplex qPCR system was determined using 10-fold serially diluted mixed genomic DNA (30−0.003 ng for each species) of the target species (cow, buffalo, and pig). The amplification plots reflected detectable Ct from all concentrations, starting from 30 ng to 0.003 ng of DNA, suggesting the assay could detect

and quantify minimum 0.003 ng of target DNA in a 20 µL of reaction mixture (Figure 4.30 a – d). The Ct values and relative standard deviation (RSD) for all the dilutions are shown in Table 4.19. RSD for all diluted DNA were less than 1.0 (0.1−0.94).

4.7.4 Quantification and Efficiency of the Tetraplex Quantitative PCR System

The quantitative detection was performed by generating separate standard curves for each of three species and IAC by plotting the Ct values against the logarithmic value of each DNA concentration (30 ng/µL that came from total genomic DNA extracted from the ternary admixture of beef, buffalo, and pork mixed in a ratio of 1:1:1). The standard curve for cow was in the range from 30 ng to 0.03 ng whereas that of buffalo, pig, and IAC were from 30 ng to 0.003 ng (Figure 4.30 e−h). Four point dilutions (30−0.03 ng) were used for the cattle quantification because a five point dilutions (30−0.003 ng) did not comply with the recommended PCR efficiency (90−110%) (Ali et al., 2012a). In all standard curves, a good linear regression were found for all measurements, wherein the regression coefficient (R2) was 0.9847, 0.9996, 0.9999, and 0.9978 for cow, buffalo, pig, and IAC, respectively, and the corresponding slopes were −3.1289, −3.1477, −3.4562, and −3.2288. The PCR efficiencies (E) were calculated using the formula described in methodology and were 108.73%, 107.82%, 94.68%, and 104.03% for cow, buffalo, pig, and IAC, respectively. These values were within the recommended limit of qPCR efficiency (90-110%) (Ali et al., 2012a).

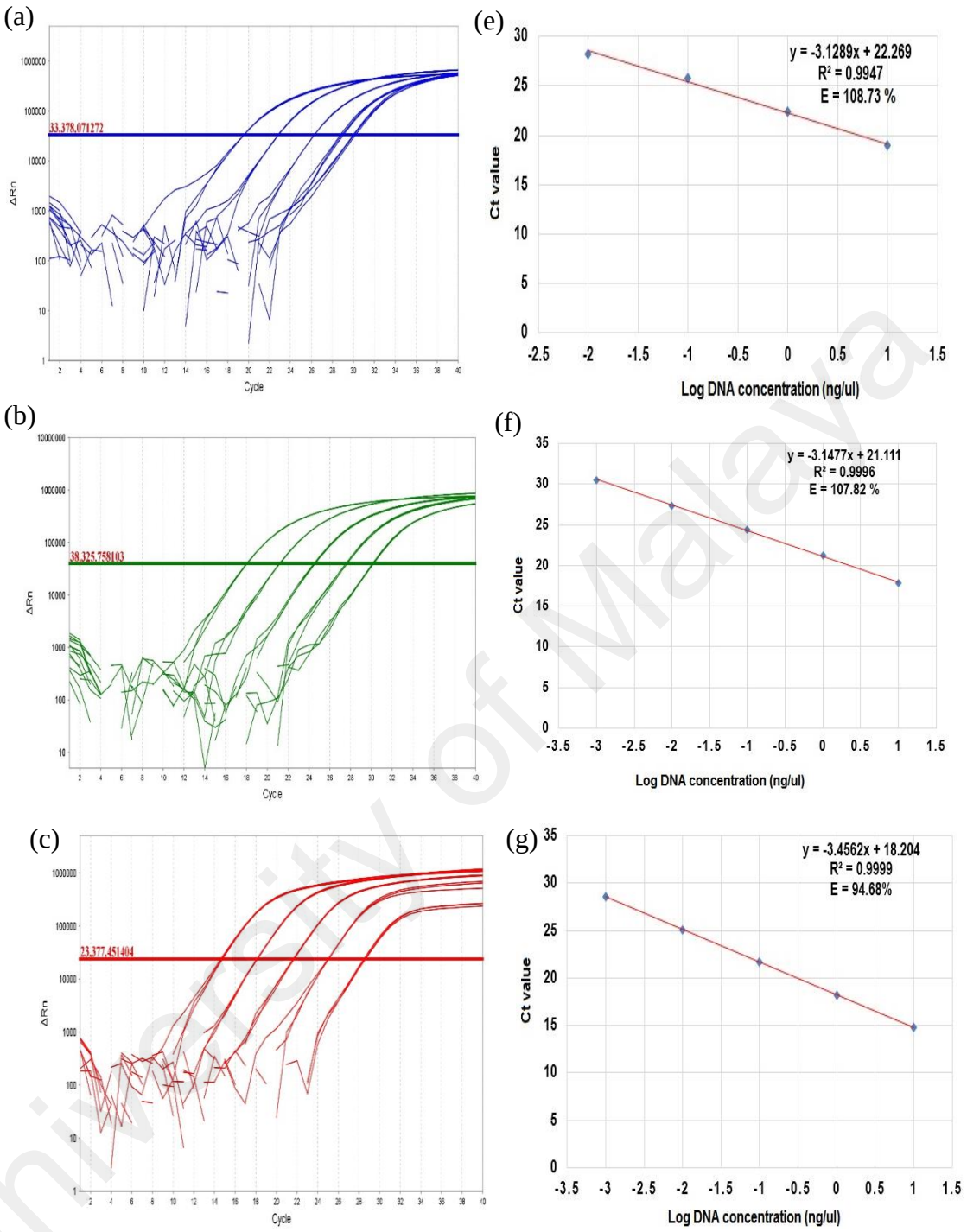
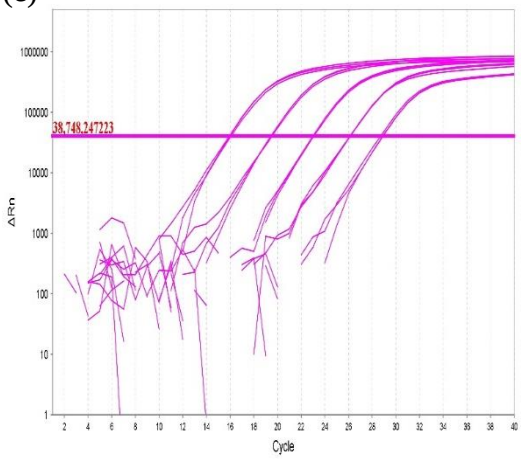


Figure 4.30: Amplification plots (a−d) and standard curves (e−h) of tetraplex qPCR products obtained from 10-fold serially diluted mixed DNA of three target species. Amplification plots and standard curves of (a and e) for beef, (b and f) for buffalo, (c and g) for pork, and (d and h) for endogenous control (IAC) specific qPCR systems, respectively.

(e)



(h)

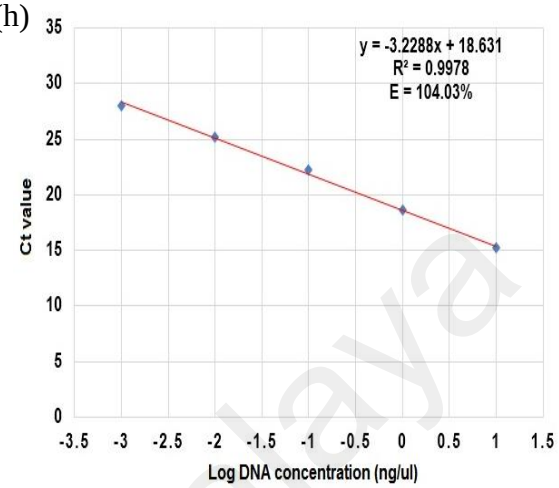


Figure 4.30: Continued

Table 4.19: Ct values of each target species obtained from the amplification plot with a 10 fold serially diluted DNA of each target species for the determination of LOD and generation of standard curves.

DNA concentration (ng)	Cow				Buffalo				Pig			
	Ct value	Mean Ct value	SD	RSD (%)	Ct value	Mean Ct value	SD	RSD (%)	Ct value	Mean Ct value	SD	RSD (%)
10	18.244 18.267 18.454	18.32	0.115	0.63	17.419 17.391 17.415	17.41	0.015	0.09	14.375 14.334 14.354	14.35	0.020	0.14
1	21.781 21.752 21.756	21.76	0.015	0.07	20.554 20.533 20.578	20.56	0.022	0.11	17.886 17.867 17.781	17.84	0.055	0.31
0.1	25.243 25.289 25.364	25.30	0.061	0.24	23.970 24.053 23.999	24.01	0.042	0.18	21.769 21.688 21.781	21.75	0.050	0.23
0.01	28.155 28.158 28.155	28.16	0.002	0.01	27.150 27.116 27.184	27.15	0.034	0.13	25.068 25.192 25.208	25.16	0.076	0.30
0.001	29.215 29.182 29.287	29.23	0.054	0.18	30.153 30.303 30.428	30.29	0.137	0.45	27.957 28.264 28.488	28.24	2.66	0.94

Note: SD, standard deviation; RSD, relative standard deviation.

4.7.5 Sensitivity and Validity of the Tetraplex qPCR Assay under Ternary and Commercial Matrixes

The The sensitivity of the developed tetraplex qPCR assay was tested to detect the level of beef, buffalo, and pork in deliberately adulterated model ternary meat admixtures (section 3.8.6) and all the species were detected until 0.1% adulteration in the ternary admixes. The Ct values of lower detectable quantity (0.1%) were 25.19 ± 0.23 to 27.68 ± 1.47 for all the three target species (Table 4.20), but the IAC constantly yielded a mean Ct between 15.63 ± 0.11 and 16.83 ± 0.21 for all level of adulterations, reflecting that the endogenous target did not change significantly with a variation in adulterations because all adulterants were eukaryotic. The inter day relative standard deviations (RSDs) were calculated from the mean Ct values of the different spiked level model meat products and were found between 0.06 and 1.2%. Only seven samples produced RSDs ≥ 1.0%, but the other 47 out of the 54 samples yielded RSDs < 1.0% (Table 4.20). These clearly demonstrated that the developed tetraplex qPCR system was very sensitive, specific, and robust and can reliably detect all the three targets from 0.1% contaminated specimens.

The tetraplex qPCR system was further validated for the analyses of processed meat products (frankfurters and meat- balls). The analysis results (Table 4.21) of the three target species revealed that the target recoveries from 10% to 0.1% spiked level were 85.90−115.3% along with a systematic error between −14.10 and +15.3% and RSD 0.61−19.40%. Thus, the maximum recovery was 115.3% for the 10% spiked pork in buffalo meatball and minimum was 85.90% for the 10% spiked beef in pork meatball product, respectively. On the other hand, maximum RSD was found in buffalo frankfurter containing 0.1% pork adulteration and minimum RSD was found in 1% adulterated pork frankfurter. When a graph was generated by plotting the recovered values (y-axis) (Table 4.21) against the reference (actual) values (x-axis) for each target, a very high correlation

coefficient (R^2 = 0.9999) was attained (Figure 4.31), confirming that the experimental values were fairly close to their actual values.

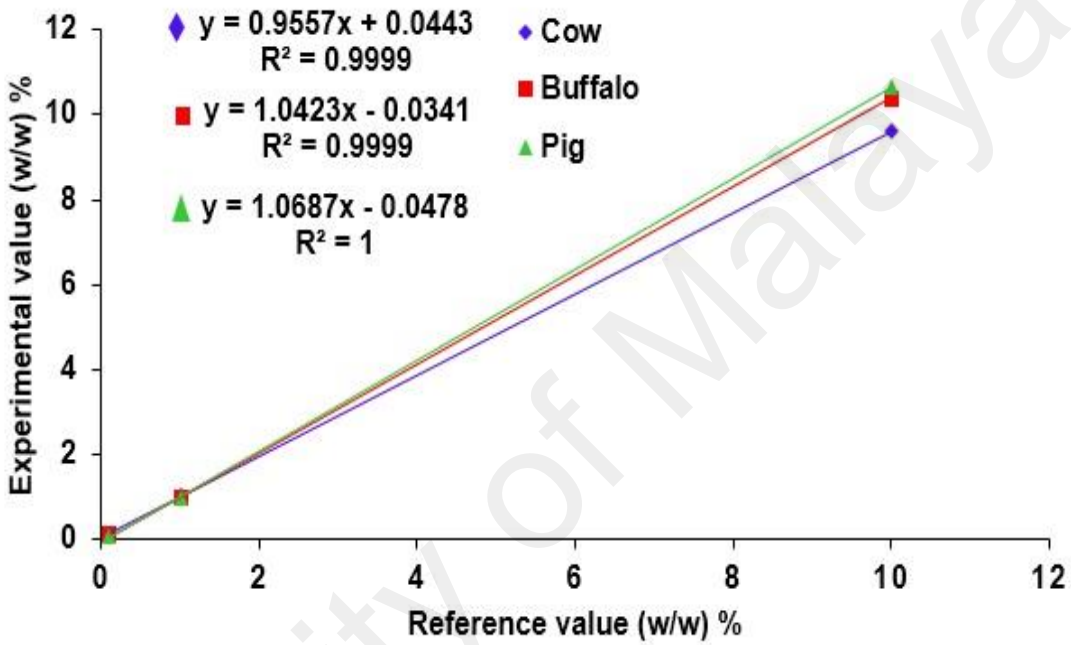


Figure 4.31: Relationship between the experimental and reference values of the tetraplex qPCR system. The experimental quantity (mean value) obtained from the tetraplex qPCR assay for adulterated (0.1, 1 and 10 %) model frankfurter and meatball of three target species plotted against the reference values that were used in their laboratory preparation.

Table 4.20: Mean Ct values and inter day RSD of different model meat products.

Products	Spike level (%)	Species	Mean Ct value			SD	RSD (%)
			Day 1	Day 2	Day 3		
Beef frankfurter	10	Beef	19.443	19.372	19.507	0.067	0.35
		Buffalo	20.805	20.992	21.142	0.168	0.80
		Pork	18.318	18.170	17.952	0.184	1.01
	1	Beef	19.180	19.152	19.124	0.028	0.15
		Buffalo	24.548	24.435	24.092	0.237	0.97
		Pork	21.546	21.705	21.736	0.101	0.47
	0.1	Beef	19.184	19.083	19.167	0.054	0.28
		Buffalo	27.583	27.240	27.315	0.180	0.66
		Pork	25.396	25.362	25.258	0.071	0.28
Buffalo frankfurter	10	Buffalo	18.291	18.221	18.285	0.038	0.21
		Beef	22.357	21.964	22.129	0.197	0.89
		Pork	17.856	18.242	18.135	0.199	1.10
	1	Buffalo	17.977	18.018	18.022	0.024	0.14
		Beef	25.536	25.207	25.116	0.221	0.87
		Pork	21.470	21.788	21.989	0.261	1.20
	0.1	Buffalo	17.926	17.988	17.946	0.031	0.18
		Beef	28.245	28.289	28.749	0.279	0.98
		Pork	24.947	25.469	25.379	0.279	1.10
Pork frankfurter	10	Pork	15.036	15.120	15.002	0.060	0.40
		Beef	22.096	22.407	22.425	0.185	0.83
		Buffalo	21.325	21.193	20.929	0.201	0.95
	1	Pork	14.800	14.806	14.788	0.009	0.06
		Beef	25.210	25.579	25.257	0.200	0.79
		Buffalo	24.114	24.293	24.495	0.190	0.78
	0.1	Pork	14.737	14.811	14.793	0.038	0.26
		Beef	28.474	28.611	28.796	0.161	0.56
		Buffalo	27.092	27.180	27.491	0.209	0.77

Table 4.20: Continued.

Products	Spike level (%)	Species	Mean Ct value			SD	RSD (%)
			Day 1	Day 2	Day 3		
Beef meatball	10	Beef	19.403	19.483	19.476	0.044	0.23
		Buffalo	21.092	21.199	21.303	0.105	0.50
		Pork	18.311	18.080	18.398	0.164	0.90
	1	Beef	19.190	19.202	19.090	0.061	0.32
		Buffalo	23.957	24.416	24.111	0.233	0.97
		Pork	21.702	21.539	21.688	0.090	0.42
	0.1	Beef	19.167	19.203	19.179	0.018	0.10
		Buffalo	27.070	27.595	27.214	0.271	0.99
		Pork	25.227	25.379	25.148	0.117	0.46
Buffalo meatball	10	Buffalo	18.177	18.203	18.314	0.072	0.40
		Beef	22.159	22.588	22.516	0.229	1.02
		Pork	17.991	17.807	18.198	0.195	1.09
	1	Buffalo	17.939	18.015	17.971	0.038	0.21
		Beef	25.113	25.479	25.207	0.190	0.75
		Pork	21.857	21.553	21.760	0.155	0.71
	0.1	Buffalo	17.903	18.014	18.003	0.061	0.34
		Beef	28.486	28.780	28.686	0.150	0.52
		Pork	25.161	24.698	24.881	0.233	0.94
Pork meatball	10	Pork	15.021	15.063	15.154	0.068	0.45
		Beef	22.626	22.404	22.407	0.127	0.57
		Buffalo	20.807	21.060	21.231	0.213	1.01
	1	Pork	14.784	14.712	14.701	0.045	0.31
		Beef	25.342	25.658	25.548	0.160	0.63
		Buffalo	24.309	24.092	24.290	0.120	0.50
	0.1	Pork	14.762	14.679	14.778	0.053	0.36
		Beef	28.715	28.214	28.314	0.265	0.93
		Buffalo	27.227	27.595	27.136	0.243	0.89

Note: SD, standard deviation; RSD, relative standard deviation.

Table 4.21: Reproducibility and recovery of the target species in model meat products.

Products	Spike level (%)	Species	Content of target determined (%)				RSD (%)	Recovery (%)	Systematic error (%)
			Day 1	Day 2	Day 3	Mean			
Beef frankfurter	10	Beef	80.01	84.31	76.33	80.21	4.98	100.26	0.26
		Buffalo	12.51	10.91	9.76	11.06	12.49	110.60	10.60
		Pork	9.27	10.23	11.83	10.44	12.38	104.40	4.40
	1	Beef	97.09	99.12	101.2	99.13	2.07	101.15	1.15
		Buffalo	0.809	0.879	1.129	0.939	17.92	93.90	-6.10
		Pork	1.079	0.971	0.951	1.00	6.88	100.00	0.00
	0.1	Beef	96.81	104.3	98.03	99.71	4.03	99.90	-0.10
		Buffalo	0.088	0.113	0.107	0.103	12.71	103.00	3.00
		Pork	0.083	0.085	0.091	0.086	4.82	91.00	-9.00
Buffalo frankfurter	10	Buffalo	78.69	82.82	79.03	80.18	2.86	100.22	0.22
		Beef	9.37	12.52	11.09	10.99	14.35	109.90	9.90
		Pork	12.61	9.750	10.47	10.94	13.59	109.40	9.40
	1	Buffalo	99.00	96.08	95.80	96.96	1.83	98.93	-1.07
		Beef	0.903	1.151	1.231	1.095	15.62	109.50	9.50
		Pork	1.135	0.918	0.803	0.952	17.71	95.20	-4.80
	0.1	Buffalo	102.8	98.21	101.3	100.77	2.32	100.97	0.97
		Beef	0.123	0.119	0.085	0.109	19.16	109.00	9.00
		Pork	0.112	0.079	0.084	0.092	19.40	92.00	-8.00
Pork frankfurter	10	Pork	82.53	78.04	84.42	81.66	4.01	102.07	2.07
		Beef	11.36	9.03	8.92	9.77	14.11	97.70	-2.30
		Buffalo	8.55	9.42	11.42	9.79	15.02	97.90	-2.10
	1	Pork	96.58	96.20	97.36	96.71	0.61	98.68	-1.32
		Beef	1.148	0.875	1.109	1.04	14.14	104.00	4.00
		Buffalo	1.112	0.975	0.841	0.976	13.88	97.60	-2.40
	0.1	Pork	100.7	95.88	97.03	97.87	2.57	98.06	-1.94
		Beef	0.104	0.094	0.082	0.093	11.80	93.00	-7.00
		Buffalo	0.126	0.118	0.094	0.113	14.78	113.00	13.00

Table 4.21: Continued.

Products	Spike level (%)	Species	Content of target determined (%)				RSD (%)	Recovery (%)	Systematic error (%)
			Day 1	Day 2	Day 3	Mean			
Beef meatball	10	Beef	82.40	77.69	78.09	79.39	3.29	99.23	-0.77
		Buffalo	10.14	9.38	8.69	9.40	7.71	94.00	-6.00
		Pork	9.311	10.86	8.788	9.65	11.16	96.50	-3.50
	1	Beef	96.39	95.54	103.75	98.56	4.58	100.57	0.57
		Buffalo	1.247	0.891	1.114	1.08	16.59	108.00	8.00
		Pork	0.973	1.084	0.982	1.013	6.09	101.30	1.30
	0.1	Beef	98.03	95.47	97.17	96.89	1.34	97.08	-2.92
		Buffalo	0.128	0.087	0.115	0.11	19.05	110.00	10.00
		Pork	0.093	0.084	0.098	0.092	7.74	92.00	-8.00
Buffalo meatball	10	Buffalo	85.53	83.92	77.37	82.27	5.25	102.83	2.83
		Beef	10.84	7.91	8.33	9.03	17.55	90.30	-9.70
		Pork	11.52	13.03	10.04	11.53	12.97	115.30	15.30
	1	Buffalo	101.8	96.29	99.44	99.17	2.79	101.19	1.19
		Beef	1.233	0.942	1.151	1.11	13.53	111.00	11.0
		Pork	0.877	1.074	0.936	0.962	10.51	96.20	3.80
	0.1	Buffalo	104.5	96.36	97.14	99.33	4.52	99.52	-0.48
		Beef	0.103	0.083	0.089	0.092	11.20	92.00	-8.00
		Pork	0.097	0.132	0.117	0.115	15.22	115.00	15.00
Pork meatball	10	Pork	83.36	81.06	76.29	80.23	4.49	100.28	0.28
		Beef	7.690	9.054	9.034	8.59	9.10	85.90	-14.10
		Buffalo	12.49	10.38	9.159	10.67	15.78	106.70	6.70
	1	Pork	97.62	102.4	103.2	101.07	2.99	103.13	3.13
		Beef	1.042	0.826	0.895	0.92	11.98	92.00	-8.00
		Buffalo	0.964	1.129	0.977	1.02	8.96	102.00	2.00
	0.1	Pork	99.06	104.7	98.00	100.58	3.58	100.78	0.78
		Beef	0.087	0.126	0.117	0.110	18.56	110.00	10.00
		Buffalo	0.114	0.087	0.122	0.108	17.03	108.00	8.00

Note: RSD, relative standard deviation.

4.7.5.1 Residual Analysis

Graph of residual verses fitted recovery values of variables for both frankfurters and meatballs of three target species (bovine, buffalo and porcine) were generated (Figure 4.32 (a-f). The random distribution of all variables were very low (within +1 to -1.5 from zero line) for beef meatballs, whereas they were relatively higher (within ± 3.0 from zero line) for beef and buffalo frankfurters as well as buffalo meatballs. On the other hand, they were within ± 2.0 for buffalo frankfurters.

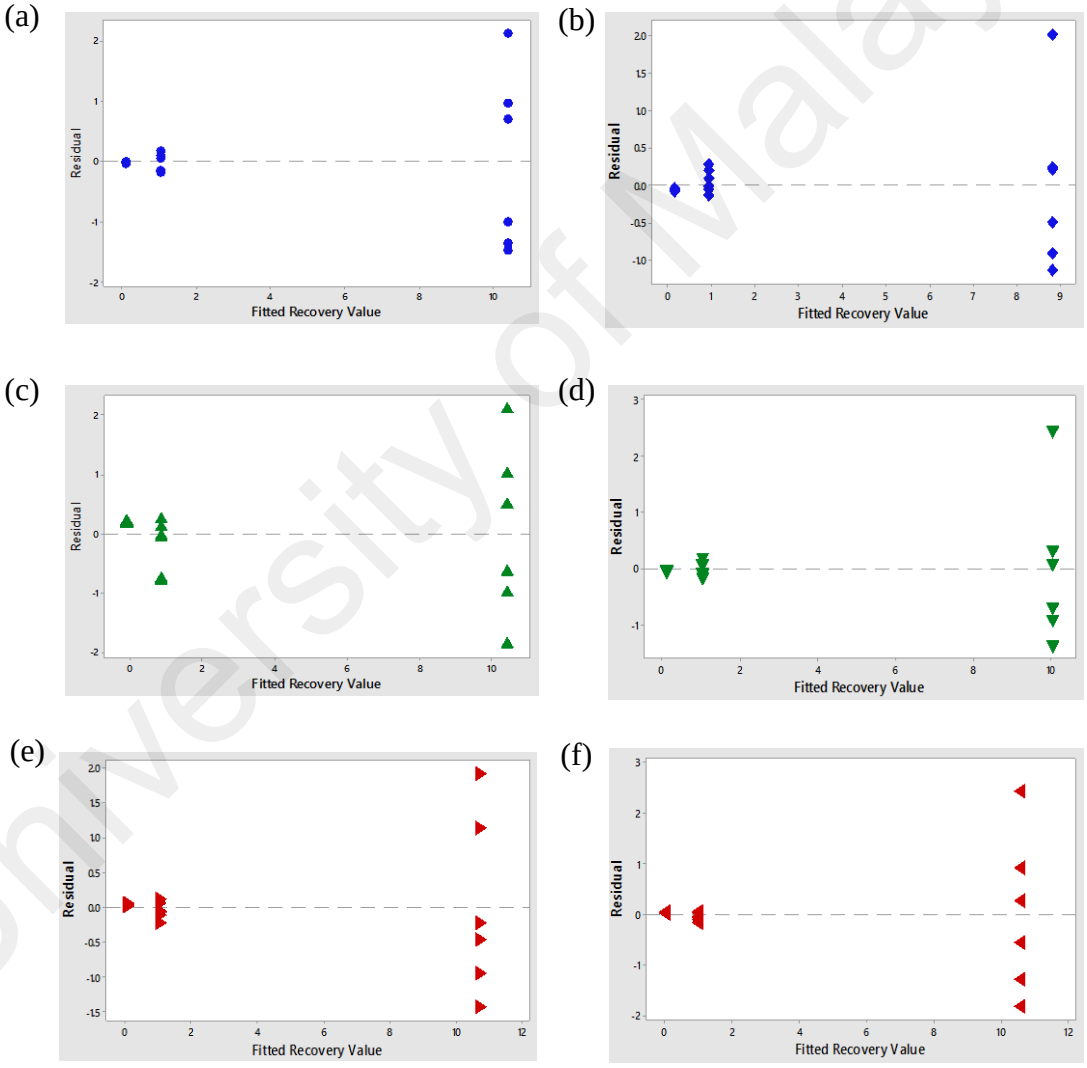


Figure 4.32: Graph of residual verses fitted recovery values of the tetraplex qPCR assays the variablely adulterated (0.1, 1 and 10%) frankfurters and meatballs of beef (a and b), buffalo (c and d) and pork (e and f), respectively.

4.7.6 Analysis of Commercial Meat Products by mqPCR

Very popular meat products namely hotdogs, meatballs and burgers were purchased from various Malaysian outlets and were analyzed using the mqPCR assay. The total analyzed products were 12 frankfurter (7 beef and 5 pork), 12 meatball (7 beef and 5 pork) and 12 burger (7 beef and 5 pork) and the results were listed in Table 4.22. The experimental results demonstrated that 71% of beef frankfurter, 100% of beef meatballs and 85% of beef burgers were adulterated with buffalo meat but no porcine DNA was found in beef products. However, all adulterated samples were found to contain both beef and buffalo but no meat products were found to contain 100% buffalo. On the other hand, all pork products were found to contain only pork and no contamination with beef and buffalo.

Table 4.22: Screening of model and commercial meat products using the developed tetraplex qPCR assay.

Sample	Adulteration		Detected species			PCR accuracy (%)
	species	%	Cattle	Buffalo	pig	
Model frankfurter						
Beef	Buffalo and Pig	10.0	3/3	3/3	3/3	100
Beef	Buffalo and Pig	1.0	3/3	3/3	3/3	100
Beef	Buffalo and Pig	0.1	3/3	3/3	3/3	100
Buffalo	Cow and Pig	10.0	3/3	3/3	3/3	100
Buffalo	Cow and Pig	1.0	3/3	3/3	3/3	100
Buffalo	Cow and Pig	0.1	3/3	3/3	3/3	100
Pork	Cow and Buffalo	10.0	3/3	3/3	3/3	100
Pork	Cow and Buffalo	1.0	3/3	3/3	3/3	100
Pork	Cow and Buffalo	0.1	3/3	3/3	3/3	100
Model meatball						
Beef	Buffalo and Pig	10.0	3/3	3/3	3/3	100
Beef	Buffalo and Pig	1.0	3/3	3/3	3/3	100
Beef	Buffalo and Pig	0.1	3/3	3/3	3/3	100
Buffalo	Cow and Pig	10.0	3/3	3/3	3/3	100
Buffalo	Cow and Pig	1.0	3/3	3/3	3/3	100
Buffalo	Cow and Pig	0.1	3/3	3/3	3/3	100
Pork	Cow and Buffalo	10.0	3/3	3/3	3/3	100
Pork	Cow and Buffalo	1.0	3/3	3/3	3/3	100
Pork	Cow and Buffalo	0.1	3/3	3/3	3/3	100
Commercial products						
Beef frankfurter	-	-	7/7	5/7	0/7	100
Pork frankfurter	-	-	0/5	0/5	5/5	100
Beef meatball	-	-	7/7	7/7	0/7	100
Pork meatball	-	-	0/5	0/5	5/5	100
Beef burger	-	-	7/7	6/7	0/7	100
Pork burger	-	-	0/5	0/5	5/5	100

CHAPTER 5: DISCUSSION

5.1 DNA Extraction

The yield of extracted total genomic DNA depends on quantity and quality of starting materials, state of samples (raw, processed, heat or chemical treated etc.), extraction kit and protocol. To get good quality DNA, I used three different types of commercial DNA extraction kit for the extraction of total DNA from three different samples such as pure meat, meat products (burger, meatball and frankfurters) and plant species. Because specific type of kit was designed for specific sample depending on the presence of proteins, ingredients etc. Furthermore, commercial DNA extraction kits offered higher yields of DNA than the conventional liquid-liquid extraction techniques due to the present of aqueous and organic phases of in this system. Moreover, commercial kits were safer for handling and there is minimal chance of damage of DNA during extraction (Al Amin, 2015).

The Genomic DNA Mini Kit was designed for the purification of total DNA, including mitochondrial DNA and genomic DNA from different animal tissues. To shorten the cell lysis time, the kit was combined with micropestle which facilitated the disintegration of homogenized tissue specimens efficiently. Proteinase K and lysis buffer were used to perform cell lysis and degradation of protein to eliminate contamination of proteins. The use of chaotropic salt enhanced the stable DNA binding to the spin column glass fiber matrix. Effective wash buffer was used to remove any contamination and finally, DNA was eluted using low salt containing TE buffer which facilitated the stabilization of storage DNA P^H (Rashid, 2015).

The concentration of extracted DNA was determined based on the absorbance reading at 260 nm and its purity was evaluated based on the ratio of absorbance at 260 nm and

280 nm. This is because 260 nm is the absorbance maxima of nucleic acids and that at 280 nm reflects the absorbance maxima of proteins. Finally, the A_{260}/A_{280} ratio provides the DNA purity indication with respect to the protein contamination.

I found the highest DNA yield in raw meat (123-269 ng/µl) and lowest in severely microwaved (700 W) samples (32-54 ng/µl) (Table 4.1). This might be due to the higher degree of denaturation and degradation of the DNA under extensive heat treatment (Ali et al., et al., 2015b). Similarly, second lowest DNA yield was obtained from the autoclaved samples (53-84 ng/µl for raw meat and 33-62 ng/µl for meat products), as prolonged heat and pressure are applied under autoclaved condition. The DNA concentration from the boiled treated samples were found relatively higher (71-125 ng/µl for raw meat and 49-91 ng/µl for meat products) than those of the microwaved and autoclaved treated samples, this might be less due to the degradation and denaturation under relatively mild heat treatment. On the other hand, the purity and the yield of DNA was comparatively higher in all pure meat samples (raw, boiled and autoclaved) than those of the meat products (raw, boiled and autoclaved); this might be due to the presence of higher amount of fat and food ingredients including salt, spices, vegetables and other food additives in the commercial meat products (Table 4.1). The absorbance ratio at A260/A280 was between 1.7 and 2.0 for all extracted DNA. Herein, the absorbance ratio at A260/A280 was between 1.8 and 2.0 for all untreated raw samples but that was between 1.7 and 2.0 for only meat products and heat treated samples. This is because some inhibitors might be present in meat products. This ensured that good quality DNA was extracted from all samples and it was suitable for PCR amplification (Nejad et al., 2014).

5.2 Development of Biomarker

The motivation of adulteration comes from a company's interest in making an on growing profit by selling a cheaper item in the name of its expensive counterparts. It

incurs a serious risk especially when an animal material is involved. According to the US Department of Agriculture, about 75% of the recently emerging infectious diseases affecting humans are the diseases of the animal origins (USDA, 2015). Certain animal materials such as bovine and porcine are also sensitive social and religious issues. Overall food falsification is a crime under the food and drug laws in most countries and its prevention is a long cherished hope. In this regards, authentication technologies play a key role by verifying the food ingredients prior to the enforcement of regulatory laws. The key purpose is not to punish the violators but to prevent the practices at its origin for the greater societal and health benefits. The adulteration of beef products with buffalo and buffalo with beef in many cases are unreported, especially when societal issues are not dominant such as in Malaysia. However, it is a matter of economic cheating and also it involves certain degree of health risk and socio-cultural outburst depending on the place and availability (Girish et al., 2013; Karabasanavar et al., 2011a, Sakaridis et al., 2013). It might also take innocent lives under special circumstances, such the killing of a man in India (Matthew, 2015). Considering the needs, I developed here six pairs of species-specific primers targeting the interspecies hyper variable and intra-species conserved regions of cytb and ND5 genes of beef, buffalo and pork (Table 3.1). The mitochondrial DNAs (mtDNA) are more focused over the nuclear ones (nDNA) for authentication studies because of its maternal origins, extra protection by mitochondrial membrane and abundance in multiple copies (Girish et al., 2004; Zha et al., 2010). In this study, additional security was ensured by targeting two different sites on two different mitochondrial genes since it is unlikely that both targets would be missing under compromised states. Additionally, all targets were kept within 146 bp in length since short-targets are thermodynamically more stable over the longer (Ali et al., 2015b). The designed two sets of primers for each of cow, buffalo and pig amplified short-length PCR products which were between 73 and 146 bp (Cocytb:120 bp, CoND5:106 bp, Bucytb:90

bp, BuND5:138 bp, Pocytb: 146 bp and PoND5: 73 bp). Biomarker targets within this range were suitable for efficient amplification and stable under extreme food processing conditions. Overall, this ensured better efficiency and accuracy of the assay to detect targets even in degraded samples (Ali et al., 2015b, Ali et al., 2015d). The success of an mPCR assay mainly depends on primer specificity and melting temperature (Tm) (Ali et al., 2015d). This is because all primers must anneal to their respective binding regions under the same set of PCR condition. In the design of species- specific primer, the oligonucleotide mismatch calculation plays critical roles since the efficiency of a PCR assay may reduce or amplification reaction may fail due to the presence of a critical mismatch in the primer binding site (Rashid, 2015a). In the present study, the developed six primer sets contained 100% matching with specific gene targets and (3-18) nucleotides (12.5-78%) mismatching with other related or non-target species, reflecting there is no probability of cross-reaction even with closely related species during PCR assays. Because the presence of single mismatch at the primer binding position might be effective to failure the PCR amplification (Rashid et al., 2015b). Furthermore, identical Tm (~ 60^0 C (57.8-60.9^0 C)) of all primers confirms that all primers would anneal only with the target template and there is very little or no possibility to anneal with any others non-target species (Table 3.1) (Razzak et al., 2015). The pairwise distances among 25 animals and 4 plant species was between 0.144 and 1.993 (Table 4.8-4.13) which was computed using the neighbour-joining method. The minimum distance was found between the beef cytb-specific 120 bp site and goat (0.144) (Table 4.8) and the maximum was between the beef ND5-specific 106 bp site and wheat (1.993) (Table 4.9), reflecting adequate genetic distances among the studied species. Moreover, the analysis of phylogenetic tree (Figure 4.1, a-f) based on genome sequences demonstrated similar findings, supporting the results of other in silico tests. In addition, the 3D plot was created from the data of mismatch of primer pairs and pairwise distance, which also support the

adequate genetic distance among the targets and non-target species (Figure 4.2, a-f). Thus bioinformatics studies ensured that there were no or very little chances for amplifying a cross-species target (Ali, et al., 2014a). To confirm the theoretical finding, PCR experiments were carried out against 27 non-target species.

5.3 PCR Assay Optimization

Optimization of the PCR reaction is a vital step to get successful PCR products. I optimized simplex PCR assay first and then duplex, triplex, tetraplex and finally hexaplex. Various components the reaction were optimized step by step. First thing considered was the reaction volume; the higher reaction volume causes higher cost but very low volume might be insufficient for the amplification of primers, particularly for the multiplex PCR assay. Therefore, I optimized in 25 µL reaction volume which was cost effective but sufficient for a multiplex PCR reaction. Buffer concentration is also important in PCR reaction. The cations of buffer neutralize the negative charged of the phosphate group of DNA template which decreases the electrorepulsive forces of between the DNA stands. As a result primer can come into contact with DNA strands easily that facilitates the annealing between them. By following the supplier instruction I used 1x buffer concentration for successful reaction. Magnesium chloride plays a critical role for success PCR amplification

Mg^{2+} is said to be a cofactor of the polymerase enzyme because it forms soluble complexes with deoxynucleoside triphosphates (dNTPs) to prepare a recognizable substrate for Polymerase. Therefore, Mg^{2+}may affect DNA polymerase activity and fidelity, specificity of PCR, denaturation temperatures of both template and PCR product DNA strand, annealing of primer and formation of primer dimer. Excess Mg^{2+} leads to nonspecific amplification due to nonspecific primer annealing, while inadequate magnesium results in decreased the yield of the expected amplified product. Thus, for

optimum activity, polymerase enzyme requires sufficient free magnesium other than that of bound with dNTP and template DNA (Markoulatos, Siafakas, & Moncany, 2002). Several experiments were repeated by changing the $MgCl_2$ concentration and finally optimized 2.5 mM concentration for simplex, duplex and triplex reactions and 3.5 and 4.0 mM were used tetraplex and hexaplex reaction. On the other hand, two different concentration (0.20 mM for simplex to tetraplex and 0.25 mM for multiplex) of the dNTPs (dATP, dCTP, dGTP and dTTP) were used to optimize the simplex to multiplex reactions. Because, concentration of dNTPs may affect the specificity, fidelity and yield of a PCR amplification, because concentration of free Mg^{2+} is affected by the amount of dNTPs. Hence Mg^{2+} binds with dNTPs. DNA polymerase fidelity reduce due to the imbalance amount of four dNTPs (Kunz & Kohalmi, 1991), whereas, excess dNTPs may result in inhibition of amplification due to increase error rate of polymerase (Kramer & Coen, 2001). Another important parameter determined experimentally was annealing temperature (Ta). The highest annealing temperature is favorable because it increased specificity by reducing non-specific binding of primers (Ali 2012c; Wu et al., 2009). Tm of all primers should be same in multiplex PCR assay because all primers are amplified in a single reaction tube with same conditions. Although Tm values of the developed six sets of primers were different (57.8-60.9^0 C) but all primers sets were able to amplify at same temperature (60^0 C), resulting the favorable for the development of mPCR assay (Figure 4.3-4.5).

After optimization the simplex PCR, two duplex PCR for Cocytb and Bucytb and CoND5 and BuND5; one triplex of Cocytb, CoND5 and BuND5; one tetraplex of Cocytb, Bucytb, CoND5 and BuND5 and finally multiplex (hexaplex) PCR of Pocytb, Cocytb, Bucytb, CoND5, BuND5 and PoND5 were optimized step by step to eliminate the possibility of forming any unwanted primer dimers or multimers (Figure 4.12 and 4.18) (Ali et al., 2015d). The novel double genes targeted mPCR system clearly amplified

targeted products [106, 138, and 73 bp (ND5 of beef, buffalo, and pork) and 120, 90, and 146 bp (cytb of beef, buffalo, and pork)]. Due to the narrow differences in the length of the targets, agarose gel electrophoresis, which hardly separate nucleic acid of less than 50 bp difference in length, could not be used to separate the amplicons for visualization (Bottero & Dalmasso, 2011). It is also a laborious technique and require rather longer separation time. Consequently, I used here a fully automated multi-capillary electrophoresis device (QIAxcel Advanced Capillary Electrophoresis System, Germany) for the separation and visualization of PCR products. This effectively enhanced sensitivity and resolution (~5 bp) and shortened analysis time, minimizing the manual handling errors and exposure to hazardous chemicals by virtue of its in-built gel matrices in a ready- to-run gel cartridge (Bottero & Dalmasso, 2011; Fajardo et al., 2010). The well separated tetraplex and mPCR products were clearly visualized in the gel image along with the electropherograms (Figure 4.12 and 4.18) for all of six targets.

5.4 PCR Assay Specificity

In the initial step of simplex PCR assay specificity test, beef primer sets (Cocytb and CoND5) were assayed against buffalo and pork DNA, buffalo primer sets (Bucytb and BuND5) with beef and pork DNA, and pork primer sets (Pocytb and PoND5) with beef and buffalo DNA as non-target to avoid cross-amplification in multiplex PCR. The next step was the cross-specificity which was performed with 27 different non-target species using 20 ng of DNA extracted from all of the tested samples. Specific PCR products [106, 138, and 73 bp (ND5 of beef, buffalo, and pork) and 120, 90, and 146 bp (cytb of beef, buffalo, and pork)] were found only from beef, buffalo, and pork, and such a product was absent from the other samples (goat, lamb, dog, cat, rabbit, monkey, donkey, chicken, duck, pigeon, quail, rat, salmon, tuna, cod, tilapia, rohu, pangas, frog, turtle, wheat, onion, garlic, ginger, and pepper). On the other hand, the use of the universal eukaryotic primers which amplified 99 bp product from all species reflected the presence of good quality

DNA in all tubes, eliminating the possibility of any false-negative detection (Figure 4.6-4.11).

After confirmation of the simplex PCR, the mPCR system was developed step by step through the duplex, triplex, and tetraplex and hexaplex (multiplex) PCR systems and cross-specificity test of the developed tetraplex and mPCR assays were performed. The developed tetraplex and mPCR system clearly amplified targeted products (73, 90, 106, 120, 138, and 146 bp) from beef, buffalo, and pork samples, and no cross-amplifications were observed in any non-target species (Figure 4.13 and 4.19), confirming that the developed tetraplex and mPCR assay was highly specific for the discriminatory detection of beef, buffalo, and pork. Triplicate assays were performed on three different days and reproducible results were obtained.

5.5 PCR Product Sequence Analysis

Although a properly designed and optimized species-specific PCR assays are often conclusive to assign specific species (Ali et al., 2015d; Karabasanavar et al., 2014) but authentication of PCR products by sequence analysis greatly increase the reliability of the PCR assay. Moreover, PCR products indicate only the presence or absence of the species but PCR products sequencing results properly confirm whether the accurate species are detected (Bevan, Rapley, & Walker, 1992). The PCR products obtained in this research were cloned prior to sequencing because they were very short-length and direct sequencing cannot derive the full length sequence of the products. The PCR products sequencing results showed that all PCR products were 100% similar with the target sequences of the specific species but the buffalo ND5 gene (Table 4.14) that showed 98.5% similarity but this value was within the acceptable limit because at least 98% sequence similarity is required for the potential species identification (Cawthorn et al., 2013). Previously, Cawthorn et al. (2013) reported that 99% sequence similarity for three

tested samples (one 'blesbok biltong' and two 'kudu biltong'). On the other hand, Natonek-Wisniewska et al. (2013) found 97.78% sequence similarity for bovine specific PCR products, whereas ovine specific products showed more than 94% similarity with *ovis* species and incase of porcine products it was more than 99% similar. Hsieh et al., (2005) also found a sequence similarity of 98 -100% for various samples. Thus, little variation in sequence similarity is a common phenomenon.

5.6 Tetraplex PCR Assay

5.6.1 LOD of Tetraplex PCR Assay

In this study, dilution method (Ali et al., 2015d) was followed to determine the sensitivity of the developed multiplex PCR system. At first, the concentration of the starting DNA was spectrophotometrically determined in triplicates at a relatively high concentration (50 ng/µL) and then sequentially diluted into 10.0, 5.0, 1.0, 0.5, 0.2, 0.1, 0.05, 0.02 and 0.01 ng/µL by adding required amount of deionized distilled water since spectrophotometry provides inconsistent data at lower concentration. Both the gel images (Figure 4.14 a) and electropherograms (Figure 4.14(b)) of the corresponding multiplex PCR products were observed from as low as 0.01 ng of DNA extracted from raw meat. After 0.01 ng, both the gel images and electropherograms became very fainted or undetectable and thus the limit of detection (LOD) of the assay was determined to be 0.01 ng total DNA extracted from raw meat. This relatively high sensitivity was attributed to the short-length amplicon (< 140 bp) targets used in this multiple PCR system (Ali et al., 2015a). This result was consistent with the previous work done in our laboratory (Ali et al., 2015b) where the LOD was 0.01 ng total DNA from pig, dog, monkey, cat and rat in a multiplex PCR with 108 – 172 bp product sizes. Safdar & Junejo, (2015) also obtained similar result (0.01%) for a multiplex PCR of ovine (119 bp), caprine (142 bp), fish (224 bp) and bovine (271 bp). In another report, LOD was found to be 0.125 ng by Bottero et al. (2003) for the identification of cow, goat and sheep in a multiplex PCR of product-

size 172 – 326 bp. On the other hand, Dalmasso et al. (2004) found 0.0025 – 0.025 ng LOD for Ruminant (Bos taurus, Capra hircus, Ovis aries) poultry, fish and pork species with 104 -290 bp target amplicons. Furthermore, Rea et al. (2001) developed duplex PCR for the detection of bovine and water buffalo milk used in making mozzarella cheese with 113 and 152 bp products and sensitivity was 0.001 ng DNA. The same primers of Rea et al. (2001) were used by Gupta et al, (2012) in a duplex PCR for the detection of beef and buffalo meat and similar result (0.001 ng) was obtained. Although the sensitivity of duplex PCR was found to be 0.001 ng, no multiplex PCR with double gene-targets has been documented for beef and buffalo meat differentiation under raw and processed states.

5.6.2 Sensitivity test of Tetraplex PCR Assay under Binary Meat Admixture

The sensitivity of multiplex PCR depends on several factors, namely target species, target gens and amplicon size (Ali et al., 2014b). In pure meat adulteration studies, the developed multiplex PCR was able to detect all targeted gene-sites (cytb and ND5) of beef and buffalo from as low as 1% of binary admixture (Figure 4.15). The bands of both adulterated buffalo (lanes 2 (1% buffalo meat adulteration with beef)) and beef (lanes 10 (1% beef adulteration with buffalo meat)) (Figure 4.13 a) were very clear and the corresponding electroferograms also demonstrated them with good resolution (Figure 4.13b). From this observation, I concluded that the developed multiplex PCR technique is able to identify less than 1% (w/w) adulterated target meat under mixed matrices (Hou et al., 2015). The detection limit of duplex PCR as documented by Rea et al., (2001) for beef and buffalo species in admix milk was up to 1%. Mane et al. (2012a and 2012b) also found 1% sensitivity of both beef and buffalo target species in simplex PCR system. Similar result (1%) was presented by Hou et al. (2015) in a mixture of three different target species (chicken, duck and goose).

5.6.3 Stability of Tetraplex PCR Assay

Extremely heat or processing treatment refer to breakdown or degradation of DNA in food products due to mechanical forces or natural decomposition (Arslan et al., 2006; Ilhak & Arslan, 2007). To study the effect of different heat treatment on target DNA breakdown pure, deliberately adulterated and commercial burgers were studied after 2.5 h autoclaving under 15-psi pressure, and raw meats were studied after 45 min of boiling, 2.5 h autoclaving under 15-psi pressure and 30 min of microwaving at 500, 600 and 700W. Boiling is the widely used traditional cooking process and a modern rapid food heating technique is microwaving. Whereas, autoclaving method is used to mimic canning and steaming process as it is applied to destroy the potential microorganisms at high pressure and temperature (~300^0 C) (Todar, 2008).

PCR products of desired targets were obtained from as low as 1% adulterated burger samples under 2.5 h autoclaving conditions (Figure 4.16). The finding was consistent with earlier studies for other species (Ali et al., 2015d). Figure 4.17 clearly shown that developed tetraplex assay successfully amplified the extracted DNA from all the thermal treated specimens including microwaving at 700W for 30 min. Microwaving at 700W is extreme heat treatment, the samples treated above 700 W for 30 min became burnt, dried out and thus not suitable for intake (Rashid, 2015a). This reflected that the tetraplex PCR technique I developed here were very stable even in degraded samples when exposed to harsh cooking conditions. Earlier studies in our laboratory, demonstrated that shorter-length PCR targets are more stable than those of longer ones (Ali et al., 2015b, Ali et al., 2015c). Thus we attributed this higher stability to the less than 140 bp length of all targets used in the multiplex PCR system. Additionally, I used here two different targets from two different mitochondrial genes (cytb and ND5) to overcome the probability of any false negative detection due to breakdown of a single gene target. It is highly unlikely that both gene sites would be lost or remain undetected due to degradation by natural

decomposition or processing treatments. To ensure the accuracy of the method, I screened here model and commercial burgers deliberately adulterated with beef and buffalo as well as beef curries purchased from various Malaysian outlets. 5 x 9 number of beef burgers were studied and none of them were found buffalo positive. On the other hand, out of seven beef curry samples, five were detected as buffalo positive. I did not find any buffalo burgers or buffalo curries in commercial markets or restaurants sold in the name of buffalo. However, a significant level (>70%) of buffalo adulteration was found in beef curries (Table 4.15). While the consumption of both buffalo and beef are allowed in Malaysia, beef price in commercial market is almost two times of buffalo meat. Thus buffalo adulteration in beef curries was for economic gain.

5.7 Multiplex PCR Assay

5.7.1 Limit of Detection of Multiplex PCR Assay

To check the sensitivity of the mPCR assay, DNA template of all target species was serially diluted from higher to lower concentration (10 ng-0.01 ng) by adding required amount of deionized water (Ali et al., 2015d) (Figure 4.20). The six distinct bands of the six PCR target amplified from 0.02 ng DNA template were observed in both the gel-view (lane 8) and electroferogram (inset), confirming the detection limit (LOD) at 0.02 ng DNA. LOD at this range was sufficient to detect any commercial frauding for profit making purposes (Razzak et al., 2015). Previously, Ali et al., (2015d) detected 0.01 ng DNA of dog, monkey and rat but 0.02 ng was found for pig and cat in a pentaplex PCR system of 108-172 bp product sizes. Zhang (2013) documented 1 pg (0.001 ng) sensitivity for a semi-nested mPCR for beef (263 bp), pork (387 bp), mutton (322 bp) and chicken (216 bp). However, in semi-nested mPCR a common primer pair is amplified at first place and the amplified product is then used as a template for the multiplex PCR. These make the assay more difficult, costlier time consuming and less trustworthy. In addition, the identical efficiency of the shortened primers for different templates often makes them

incapable to detect an accurate species (Ali et al., 2015d). Furthermore, Kitpipit et al. (2014) obtained 7-21 fg LOD for beef, Pork, lamb, chicken, ostrich and horse with amplicon sizes of 100-311 bp. On the other hand, Luo et al. (2008) detected 0.1-0.2 ng DNA in a mPCR assay for cattle, pig, sheep and chicken containing 149-274 bp product sizes. Additionally, a duplex PCR identified beef (113 bp) and buffalo (152 bp) milk and meat from 0.001 ng DNA (Rea et al., 2001; Gupta et al., 2012). Thus the variation in sensitivity is a common phenomenon in mPCR and it depends on several factors such as target gene, target species, amplicon size and quality of the source material (Hou et al., 2015).

5.7.2 Sensitivity and Stability Test of Multiplex PCR Assay

Although several assays are reported for bovine, buffalo and Porcine identification, the most of the reported mPCR assays have not been optimized and tested for adulteration detection under commercial matrices and extensive food processing conditions. This novel mPCR assay was optimized and validated for the analysis of a very popular meat product, meatball and frankfurter under raw and heat treated condition to evaluate the reliability and accuracy of the method. It positively detected six targets for beef, buffalo, and pork from up to 0.1% adulterated and severely autoclaved model meatballs and frankfurter (Figure 4.21 and 4.22), reflecting the sensitivity and discriminatory attributes of the novel PCR assay. More recently, Razzak et al. (2015) documented a sensitivity threshold of 0.1% for adulterated meatball, burger and frankfurter products under raw states but up to 1% for 2.5 h autoclaved samples. Safdar & Junejo (2015) also reported a 0.1% limit of detection (LOD) for the identification of ovine, caprine, fish, and bovine material using a tetraplex PCR assay involving 119−271 bp amplicons in heat-treated (133 °C at 300 kPa for 20 min) mixed meat. In another report, Safdar et al. (2014) documented 0.01% LOD for the identification of horse, soybean, poultry and pork with 85−212 bp amplicon targets. Hou et al. (2015) reported a sensitivity limit up to 1% for

chicken, duck and goose boiled at 100^0 C. In simplex PCR, Karabasanavar et al. (2014) identified 0.1% adulterated pork under raw states. Mane et al. (2012) documented less than 1% autoclaved beef in mixed states. However, instead of using processed samples, most of them used raw meat.

As like as tetraplex PCR assay, the mPCR assay also validated under extreme thermal treated meat samples namely boiled (98^0 C for 90 min), autoclaving (121^0 C and 15-psi for 20 min and 2.5 h) and microwaving (500, 600 and 700 W for 30 min) and the developed mPCR assay successfully amplified the DNA extracted from the all heat treated samples, even from the sample treated at 700 W for 30 min (Figure 4.23).

To the best of our knowledge, no other studies have tested mPCR assay under extreme food processing conditions such as boiling (90 min), autoclaving (2.5 h) and micro-oven cooking (700 W for 30 min) (Figure 4.23), although simplex PCR assays have been documented under harsh processing condition (Ali et al., 2015b, Ali,et al., 2015c; Karabasanavar et al., 2011a, Karabasanavar et al., 2011b). In earlier report, it was scientifically proven that the stability of the PCR assay under extensive processing atmosphere largely depends on the amplicon sizes; longer targets break down before the shorter ones (Ali et al., 2015b; Ali et al., 2015c). This study has carefully addressed this point and kept amplicon lengths between 73 and 146 bp; additionally, double gene sites were used as targets for each species to complement a potential missing target. Therefore, this novel mPCR assay offered better reliability but equivalent sensitivity compared to those of other published reports. In addition, the specialty of our assay was double gene target, short amplicon length, exceptional stability and sufficient sensitivity under raw, admixed and processed states. A double targeted PCR assay is more reliable and trustworthy since alternative targets can compensate the detection of a missing target under the state of decomposition.

5.7.3 Commercial Product Analysis under mPCR Assay

The motivation of the substitution of an expensive meat with its cheaper counterpart comes with the inclination of a company to have more sales and better profit, and instead of raw meat, adulteration could be skillfully manipulated in processed meat products (von Bargen et al., 2013). Because meatball and frankfurter are very popular and consumed widely all over the world, I have screened 45 and 20 halal branded beef meatball and frankfurters, respectively in Malaysian markets (Table 4.17). It would be noteworthy here that no buffalo meatball and frankfurter products were found in the Malaysian markets; that is, all were labeled as beef products. However, all the tested beef frankfurters were found as both beef and buffalo positive; this indicated that all beef frankfurter products in Malaysia was buffalo adulterated. On the other hand, beef meatballs were detected both beef and buffalo for 80% samples and total replacement of beef with buffalo in other 20% (Table 4.17).

I also checked chicken and pork meatballs and frankfurters, but none of them were beef and buffalo positive; this was probably because the prices of beef and buffalo are higher than those of chicken and pork. Although both beef and buffalo are permitted by the Halal Authority of Malaysia, beef meatball and frankfurter were adulterated with buffalo for gaining unjustified profit since the buffalo meat is cheaper than beef in Malaysia. However, as per the European guideline, fake labelling in food is a crime and it must be prevented (Przyrembel, 2004). However, because of the strict monitoring of haram species, Halal certified buffalo adulterated beef meatballs and frankfurters as well as chicken frankfurters were found free from any non-Halal porcine materials.

Although several PCR assays are proposed for the beef and buffalo differentiation (Karabasanavar et al., 2011a; Mane et al., 2012b), none of them were tested under commercial matrices despite having the risk of PCR inhibition by multiple ingredients

present in commercial products (Bottero et al., 2002; Di Pinto et al., 2005). Previous reports analyzed only model meat products such as kabab, patty, and meat block using simplex PCR systems for beef and buffalo, which incurs additional cost and time due to the use of separate assays for each species (Mane et al., 2012a; B. G. Mane et al., 2012b). Although several reports were documented for the analysis of meatball, streaky bacon, frankfurter, and burger model products for the identification of pig species (Ali et al., 2012c; Erwanto, Abidin, & Rohman, 2012), all of those were simplex PCR assays.

5.8 Authentication of PCR products by RFLP Analysis

Species-specific PCR assay is often conclusive (Ali et al., 2015d), but it has yet to be considered a definitive analytical method because of certain "hard-to-control" features of the amplification process (Focke et al., 2010; Yang et al., 2005). For example, it sometimes produces artifacts due to contamination by alien DNA at a minute scale (Doosti et al., 2014; Yang et al., 2005), but these ambiguities or doubts could be eliminated by the verification of the amplified product through at least one of three different methods, namely, PCR-RFLP assay, probe hybridization, and target product sequencing (Maede, 2006). Probe hybridization is an attractive technique because it can detect multiple species in a single experimental run through the use of multiple labeled probes (do Nascimento et al., 2010), but this procedure requires purified DNA and is also laborious, expensive, and time- consuming (Rashid et al., 2015b). In contrast, DNA sequencing is a more efficient and reliable tool, but it requires an expensive laboratory setup and is often not suitable for the analysis of processed food under complex matrices (Girish et al., 2004; Mafra et al., 2007) because of the coextraction of the food ingredients that often bring errors into the final results (Albers, Jensen, Bælum, & Jacobsen, 2013). In contrast, the PCR-RFLP assay can overcome all of these limitations and has been widely used to authenticate the original PCR product amplified from a particular gene fragment (Park et al., 2007; Sharma et al., 2008). It comprises the generations of a specific

fragment profile through restriction digestion with one or two endonucleases. A carefully selected restriction endonuclease cleaves the PCR product at specific recognition sites, producing a set of DNA fragments of different lengths that could be separated and visualized by gel electrophoresis;48 thus, it distinguishes the artificial PCR product from the original through the analysis of the restriction fingerprints (Doosti et al., 2014; Murugaiah et al., 2009). In this research, firstly, each target was digested individually with an appropriate RE (Table 3.9) to study its different restriction profile in order to eliminate any ambiguities that may arise from the final tetraplex PCR products that were the mixture of four different amplicons (Figure 4.24). Then, tetraplex PCR products of beef and buffalo were digested simultaneously with three restriction enzymes as cited in section 3.7.1.2, and clear fingerprints were obtained for each of the four different targets (Figure 4.24 and Table 3.9). Three different restriction enzymes (*FatI, EciI,* and *AluI*) were used for the digestion of four PCR products (two beef (cytb and ND5) and two buffalo (cytb and ND5)). *FatI* enzyme digested two products (buffalo cytb (90 bp) and beef ND5 (106 bp)) and other two products, beef cytb (120 bp) and buffalo ND5 (138 bp) were digested by *EciI* and *AluI,* respectively (Table 3.9). A total of eight restriction fragments (8, 19, 40, 45, 50, 75, 87, and 130 bp) were generated after digestion of four simplex PCR product with RE. The generated seven fragments were clearly visualized and 8 bp fragment was not detected because it was below the lower resolution limit of the instrument (≤15 bp). When tetraplex PCR products were digested using the same three enzymes (*FatI, EciI,* and *AluI*) in a single experimental tube which generated a RFLP patterns that were comprised of a total of seven fragments (19, 40, 45, 50, 75, 87, and 130 bp). The finding was consistent with RFLP profile of simplex PCR products, indicating that the developed tetraplex PCR also amplified the same target region as simplex PCR assay. The sizes of the digested fragments were the same as the sizes obtained from the theoretical RFLP analysis using NEBcutter software (Table 3.9). Thus, experimental

results were supported the theoretical RFLP analysis, indicating that developed PCR systems amplified exact target sites.

The developed mPCR-RFLP assay was evaluated for the screening of commercial beef and buffalo frankfurters under raw, boiled, and autoclaved states. Dummy frankfurters were deliberately adulterated, and their restriction digestion patterns were studied (Figure 4.25). The digest of all samples (raw and heat treated) clearly presented the signature fingerprints of 7 fragments (Figure 4.25), reflecting that variations in food processing treatments cannot affect the stability of any of the four biomarkers developed in this study; in other words, this novel mPCR-RFLP assay was sensitive, reliable, and robust for the discriminatory detection of beef and buffalo in processed foods.

To authenticate by RFLP, the two pork (Pocytb and PoND5) simplex PCR products were digested individually with CviKI-1 and FatI RE, respectively, because in silico studies using NEBcutter software demonstrated overlapping fragments with beef and buffalo. After digestion, the PoND5 PCR product (73 bp) generated 52 and 21 bp fragments, while Pocytb PCR product (146 bp) produced 3 fragments of 80, 45, and 21 bp (Figure 4.26). Similar products were found from boiled (98 °C for 90 min) and autoclaved (121 °C at 15-psi for 2.5 h) pork frankfurters. The restriction digestion maps of different heat-treated (boiled and autoclaved) samples were similar to those from the raw sample (Figure 4.27). Therefore, the finding indicated that as like as the beef and buffalo biomarkers, the stability of the pork biomarkers were not affected by different food processing treatments. Thus the developed hexaplex PCR assay was highly stable, reliable and very sensitive tool for the identification and differentiation of bovine, buffalo and porcine materials in severely degraded food products.

Previously, Haider et al., (2012) reported a PCR-RFLP assay with a 710 bp amplicon that was amplified using common primer pairs for the cow, chicken, turkey, sheep, pig,

buffalo, camel, and donkey. Girish et al. (2005) also documented a PCR-RFLP assay with 456 bp amplicon length for the detection of Goat, Sheep, Cattle and Buffalo. Recently, Kumar et al. (2014) proposed a RFLP pattern with a 609 bp target to discriminate cattle, buffalo, goat, sheep and pig. In addition, Erwanto et al. (2012) demonstrated a PCR-RFLP technique for a 359 bp product. On the other hand, Verkaar et al. (2002) introduced a PCR-RFLP assay for the identification and discrimination of bovine species with 271, 651, 604 and 822 bp products containing four sets of primers. A PCR-RFLP assay with universal primer pair of 360 bp amplicon sized was used for the detection of ten common meat species (cow, buffalo, pig, deer, chicken, goat, duck, turkey, rabbit and ostrich) (Wong, Lim, & Chua, 2010). Furthermore, pork species also identified by PCR-RFLP assay with 109 bp target (Ali et al., 2012c). However, such long targets (271−822 bp) are more prone to break down and thus would definitely lose their applicability for the analysis of highly processed foods. In contrast, here I reported a double gene site and short amplicon length (≤146 bp) mPCR-RFLP and systematically proved its reliability and sensitivity under raw, boiled (98 °C for 90 min), and autoclaved (121 °C and 15 psi pressure for 2.5 h) atmospheres for differential identification of beef, buffalo, and pork in pure, admixed, and frankfurter formulation.

5.9 Real-time PCR Assay

5.9.1 Multiplex Real-time PCR System

Design of specific primers and probes were the key step in the development of mqPCR system for bovine, buffalo and porcine species detection because it was necessary to ensure that all the primers and probes must have the same or very closely related melting temperatures (Tms) so that they can anneal to their specific partner sites in template DNA under the same set of PCR conditions (Cheng et al., 2014). The Tms of three primer sets were (57.8- 61.0^{0}C) which annealed to the primer binding sites at 60^0 C and Tms of the probes (68.5-70.70^{0}C) were 8-10^{0}C higher than that of the primers to facilitate the

preferential binding of the probes prior to the annealing of the primers to the template (Arya et al., 2005). The multiple amplicons were discriminated in the same reaction tube through three different fluorescent reporter dyes (section 3.8.1 and Table 3.12). The Ct values of tetraplex qPCR assay were Ct = 18.74 ± 0.04, 17.75 ± 0.06, 14.80 ± 0.05, and 15.14 ± 0.05 that nicely matched with the qPCR Ct for cow, buffalo, pig, and IAC, respectively, effectively confirming that there were not any significant variation of Ct values when the platforms were changed from single to multiplex. The use of endogenous system in the qPCR assay eliminated any false negative detection as well as helped in accurate quantification of target. It also indicated whether there is any effect of inhibitors and reagents in the reaction mixture (Rojas et al., 2011). Moreover, the presence of endogenous control was mandatory, particularly for the analysis of extremely processed food samples since the extracted DNA might be of low quality and degraded. Furthermore, the endogenous system play a key role to a verify qPCR assay if any amplification variations was occurred with species specific biomarkers due to the variation in template DNA concentration, purity of extracted DNA, degradation of DNA and the presence of PCR inhibitors (Soares et al., 2013). Therefore, factual error between the unknown samples and standards can be eliminated by the comparison of endogenous system and species-specific assay signal generated from samples (Rojas et al., 2010).

5.9.2 Specificity of the Multiplex Real-time PCR System

NCBI BLAST analysis results demonstrated that the designed primer pairs and probes had completely identical sequences with target species and sufficient mismatch with the other species. Alignment of primer sets and probes with target and non-target species (commonly used in meat products) using MEGA5 software showed 100% sequence similarity with the target species and multiple nucleotide mismatches (3-18 nt in primers and 3-25 nt in probes) with other related or non-target species (Table 4.2-4.7). From the in silico specificity analysis, it can be concluded that there were no or very little possibility

for amplifying the non-target species in a practical PCR experiments. Because the existence of a single mismatch at the primer annealing position may reduce the PCR efficiency or causes false or no amplification (Wu et al., 2009). Finally, the practical specificity of the mqPCR system was conducted with 30 ng of DNA extracted from fresh muscle tissues of three target species (cow, buffalo and pig) and 25 non-target species (lamb, goat, cat, dog, pigeon, chicken, quail, duck, rat, monkey, rabbit, donkey, tilapia, tuna, rohu, salmon, cod, pangas, turtle, frog, onion, ginger, wheat, garlic, and pepper) on three different days in triplicates. The amplification profile (Figure 4.29) clearly showed that the mqPCR system amplified only three target species (cow, buffalo and pig) with the Ct values of 18.84±0.06, 17.86±0.03 and 14.83±0.08, respectively (Table 4.18) and only background florescence were provided from non-target species within 40 cycles, confirming the absence of any cross-amplifications. On the other hand, this study used the endogenous PCR system (eukaryotic 18S rRNA) to eliminate any false negative amplification. The endogenous system amplified eukaryotic target from all targets and non-target species with the Ct values between 15.61 and 18.50 (Table 4.18) reflected that good quality DNA template was present in all tubes (Figure 4.29). Thus the developed mqPCR system effectively amplified only target species and no cross-amplifications were observed, reflecting the high specificity of the technique.

5.9.3 Limit of Detection and Efficiency of the Multiplex Quantitative PCR System

Ten fold serially diluted genomic DNA (30 to 0.003 ng) from each of the target species (cow, buffalo and pig) were used to determine the LOD of the assay and amplification plots clearly demonstrated that the system amplified up to 0.003 ng DNA with detectable fluorescence signals for all targets, suggesting the assay could detect and quantify minimum 0.003 ng target DNA (Figure 4.30 a – d). RSD for all diluted DNA were less than 1.0 (0.1−0.94) (Table 4.19), indicating that minimum variation between the replicates were present in the developed mqPCR assay. Previously, Cheng et al. (2014)

reported an mPCR system for the identification of duck, pig and chicken, wherein the LOD was 0.15 ng DNA for each species. On the other hand, it was 0.32 ng DNA for beef, pork, chicken and turkey as documented by Koppel et al. (2008). Recently, our laboratory also detected 0.0017 ng DNA by a duplex SYBR Green PCR for Malayan Box Turtle (Asing et al., 2016a). Thus LOD might vary from species to species and samples to samples but 0.001 ng detectable limit of the present assay made it highly sensitive for the adulteration authentication.

For quantitative detection standard curves of all target species were generated by plotting the Ct values against the logarithmic value of each DNA concentration. The standard curve of buffalo and pig were constructed from five point dilutions (30-0.003 ng), whereas four point dilutions (30-0.03) were used for the generation of cow standard curve. Because a five point dilutions (30-0.003 ng) did not comply with the recommended PCR efficiency (90-110%) (Safdar & Abasıyanık, 2013). The quantification of 0.03 ng DNA was sufficient to detect any commercial frauding for profit making purposes (Razzak et al., 2015). In fact, a good linear regression were found in the standard curves for all measurements, wherein the regression coefficient (R^2) was 0.9847, 0.9996, 0.9999, and 0.9978 for cow, buffalo, pig, and IAC, respectively, and the corresponding slopes were −3.1289, −3.1477, −3.4562, and −3.2288. The PCR efficiency were found to be 108.73%, 107.82%, 94.68%, and 104.03% for cow, buffalo, pig, and IAC, respectively. These values were within the recommended values (90-110%) (Ali et al., 2012a) and thus, the generated standard curves and mqPCR systems were suitable for the quantitative determination of the target species contribution from mixed meat samples. The findings were supported by Cheng et al. (2014) in which the mqPCR efficiencies were 104.38, 91.75 and 97.46% for chicken, duck and pig species, respectively. Similarly, Iwobi et al. (2015) found the efficiencies of their mqPCR system for beef and pork at 101.1% and 91.6%, respectively.

5.9.4 Sensitivity and Validity of the tetraplex qPCR Assay under Ternary and Commercial Matrices

Sensitivity of the PCR system is a key factor for the authentication of processed food products. Deliberately adulterated model ternary meat admixtures of beef, buffalo and pork (10, 1 and 0.1%) were prepared (section 3.8.7) to evaluate the sensitivity of the mqPCR method. All the species were detected until 0.1% adulteration in the ternary admixes with Ct values of 25.19 ± 0.23 to 27.68 ± 1.47 for all the three target species but the IAC constantly yielded a mean Ct between 15.63 ± 0.11 and 16.83 ± 0.21 for all level of adulterations, reflecting that the endogenous target did not change significantly with a variation in adulterations because all adulterants were eukaryotic. These clearly demonstrated that the developed mqPCR system was very sensitive, specific and robust and can reliably detect all the three targets from 0.1% contaminated specimens. Cheng et al., (2014) developed an mqPCR system for the detection of pig, chicken and duck with the sensitivity of 1% for all target species in ternary mixture. Recently, the sensitivity of the mqPCR in binary admixture was found to be 0.5% spiked level of pork in beef background (Iwobi et al., 2015). The same sensitivity (0.5%) was also found by Dooley et al., (2004) in a TaqMan real-time PCR assays for the detection of beef, pork, turkey, chicken and lamb. More recently, Fang and Zhang (2016) established a qPCR assay for the detection of murine species with sensitivity of 0.1% murine adulteration in meat admixtures.

The mqPCR system was further validated for the analyses of processed meat products (frankfurters and meatballs). The analysis results (Table 4.20) of the three target species revealed that the target recoveries from 10% to 0.1% spiked level were 85.90−115.3% along with a systematic error between −14.10 and +15.3% and RSD 0.61−19.40%. Thus, the maximum recovery was 115.3% for the 10% spiked pork in buffalo meatball and minimum was 85.90% for the 10% spiked beef in pork meatball product, respectively.

On the other hand, maximum RSD was found in buffalo frankfurter containing 0.1% pork adulteration and minimum RSD was found in 1% adulterated pork frankfurter. When a graph was generated by plotting the recovered values (y-axis) (Table 4.21) against the reference (actual) values (x-asis) for each target, a very high correlation coefficient (R^2 = 0.9999) was attained (Figure 4.31), confirming that the experimental values were fairly close to their actual values. Druml et al. (2015) found 40.9% systematic error and 12.9% RSD for 2% adulteration and Asing et al. (2016a) found 23.10% systematic error and 1.69% RSD and for 0.1% contamination. Thus the systematic error between -19.0 and +23.8% and RSD 2.04 and 22.86% of this assay was within the acceptable limits of the published reports.

5.9.4.1 Residual Analysis

Residuals are differences between the actual or predicted and the measured values from a set of variables. They determine the experimental errors by subtracting the experimental value from the predicted value (Ali et al., 2012a). Therefore, the graph of residual verses fitted recovery values of variables for both frankfurters and meatballs of three target species (bovine, buffalo and porcine) were generated (Figure 4.32 a-f). The random distribution of all variables were very low (within +2.0 to −2.0 from zero line) for frankfurter, whereas they were relatively higher (within +3.0 to −2.0 from zero line) for meatball products. These distributions of residuals indicated a good precision and accuracy of the developed mqPCR system for the measurement of 0.1-10% adulteration of the three target species in meat products (Ali et al., 2012a).

5.9.5 Analysis of Commercial Meat Products by mqPCR

The motivation of the replacement of an expensive meat with its cheaper counterpart comes with the inclination of a company to have more sales and better profit and therefore instead of raw meat, adulteration practices are skillfully manipulated in processed meat

products. Bovine, buffalo and porcine adulterations in food chains have enormous threats to public health, religions, cultures, and economy. Since, hotdogs, meatballs and burgers are very popular meat products and very widely consumed all over the world, 12 frankfurter (7 beef and 5 pork), 12 meatball (7 beef and 5 pork) and 12 burger (7 beef and 5 pork) products were procured from various Malaysian outlets and were analyzed using the tetraplex qPCR assay (Table 4.22). The experimental results revealed that 100% of beef meatballs, 85% of beef burgers and 71% of beef frankfurter were adulterated with buffalo meat but no porcine DNA was found in beef products. Surprisingly no meat products were found to contain 100% buffalo but all adulterated samples were found to contain both beef and buffalo, strongly suggesting that these adulterations were for the purpose of economic gain. On the other hand, all pork products were found to contain only pork and no contamination with beef and buffalo. These clearly reflected that buffalo substitution in beef products are very rampant in Malaysia and it is mainly done for economic gain since buffalo is cheaper than beef in Malaysian Markets but fraud stars are not mixing any porcine in halal products because of the strict monitoring of halal status in by the Malaysian government.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

Fraud labelling of meat species in food products is mainly done to realize additional economic gain by selling lower valued ingredients in place of a higher priced one. These malicious practices not only cheat customers economically but also pose huge adverse impacts to our public health, religious faiths and cultures, endangered wild species and reputation of our businesses. These practices must have to be stopped and regulatory bodies must need a trustworthy and convenient technique to reveal accurate product information. Bovine, buffalo and porcine adulteration in food chains are very common across the globe and definitely a great threat to our public health, religions, cultures, and economy. It is also a very sensitive issue because many religions do not allow their followers to consume porcine and bovine products. Several duplex PCR systems for the detection of beef and buffalo or beef and pork have been documented; but to the best of our knowledge, no methods have been proposed for reporting all the three species, namely, bovine, buffalo and porcine in a single assay platform.

This study has addressed the above knowledge gap by developing and validating a multiplex PCR (mPCR) system involving double-sites of two different mitochondrial genes (cytb and ND5) of cow, buffalo and pig species for the first time. This novel mPCR assay is outstanding because it ensured extra security through targeting two different sites of two different genes that are less likely to break down even under the states of decomposition. Furthermore, the alternative target can complement the detection of a potential missing target that might be degraded during food processing. Additionally, the detection of multiple targets in a single assay is highly promising because it definitely saves analytical cost and time. The assays were evaluated for specificity, stability and

sensitivity under raw, admixtures and commercial meat products including burger, meatball, frankfurter and beef curry.

The purity of the DNA extracted from raw and processed meat and commercial meat products were of good quality and various commercial kits had to be used for an optimum yield. Unlike pure meat, commercial meat products contain spices, food additives and vegetables and so a need different kit was necessary to get good quality DNA.

Species-specific PCR assay was developed step by step because it is a simple and low-cost technique that could be performed in most laboratories and also it is often conclusive for the speciation of different meat. So, at first primers specificities were confirmed through simple PCR and eventually multiplex PCR for all the six targets were optimized and validated against the 3 target and 27 non-targets species. A universal eukaryotic primer set were used to co-amplify a 99 eukaryotic control from all tubes, eliminating the possibility of any false-negative detection.

At first, a tetraplex PCR system was optimized for the simultaneous detection and differentiation of cow and buffalo species and a cross-specificity test was performed against 21 non-target animal and 5 plant species; it yielded PCR products only from the beef (106 and 120 bp) and buffalo (90 and 138 bp) targets and no products from non-targets, indicating the high specificity of the tetraplex PCR assay. The stability and sensitivity was confirmed under extensive autoclaving, boiling and microwaving treatments for pure meat and meat-based products such as burgers. In addition to double targets, all targets of both species were kept between 90 and138 bp which offered additional stability because short targets are more stable than the longer ones. The developed tetraplex PCR system was validated by the screening beef curries and beef burgers that revealed that 71% beef curries in Malaysia were adulterated by buffalo meat.

After validating a tetraplex PCR assay for beef and buffalo, a hexaplex PCR (mPCR) assay targeting double gene sites for each species were developed for the discrimination of bovine, buffalo and porcine materials in food chain. This multiplex system amplified all the target gene (Cytb and ND5) sites accurately, reflecting conformity with the simplex PCR system. Change of operators and laboratories did not change the findings. Cross-species were tested under pure, mixed and commercial matrices under various processed treatments but no significant variations were detected, confirming the assay fidelity. The sensitive of this hexaplex PCR assay was 0.02 ng DNA or 0.1% meat under mixed matrices. The method was validated for the screening of commercial meatball and frankfurter products and operationally feasibility was found for the detection of trace amount of bovine, buffalo and porcine materials in food chain. Thus, the novel assay demonstrated sufficient merits to be used by regulatory bodies for beef, buffalo, and pork authentication even under the state of degraded specimens.

Market survey revealed about 80% of beef meatballs are adulterated with buffalo and about 20% are totally replaced with buffalo. However, the beef frankfurters were found to contain both beef and buffalo positive in 100% tested samples; this indicated that beef products adulteration with buffalo is rampant in Malaysia. On the other hand, chicken and pork frankfurters were not positive for beef and buffalo; this was probably because the prices of beef and buffalo are higher than those of chicken and pork in Malaysia. However, no porcine material was fond in Halal branded beef and chicken products.

Authentication of PCR products is very important to be confirmed that authentic targets were amplified. Two types of authentication schemes were applied; sequencing and RFLP analysis. The amplified products were purified and cloned prior to sequencing the results showed that all PCR products were 100% identical with the specific target sequences 98.5% similar but it was within the acceptable range. In the second way, PCR

products were digested by four different restriction enzymes such as *AluI, EciI, FatI,* and *CviKI-* that confirmed the authentic molecular fingerprints for the target species. The tetraplex PCR products of beef and buffalo were digested simultaneously with three restriction enzymes and clear fingerprints were obtained for each of the four different targets. The tetraplex PCR-RFLP assay was validated for the screening of deliberately adulterated commercial beef and buffalo frankfurters under raw, boiled, and autoclaved states and signature fingerprints were obtained. Thus it was confirmed that this novel tetraplex PCR-RFLP assay was sensitive, reliable, and robust for the discriminatory detection of beef and buffalo in processed foods. On the other hand, porcine PCR products were digested individually with *CviKI*-1 and *FatI* RE, because they produced overlapping fragments for beef and buffalo; it was proven that like beef and buffalo biomarkers, pork biomarkers was also stable under food processing treatments.

Although our conventional mPCR and mPCR-RFLP assays successfully identified bovine, buffalo and porcine materials in food chain, they cannot quantify how much adulterant was used. Therefore, a short amplicon length TaqMan probe multiplex (tetraplex) real-time PCR assay was developed for the quantitative detection of beef, buffalo and pork in a single assay platform. It was a greatly reliable assay for the quantitative determination of bovine, buffalo, and porcine materials under any matrices because specific targets were detected firstly, by species-specific primers and secondly, by the TaqMan probes, confirming a double checking satisfaction. Thirdly, the shorter length of the targets offered additional stability even under the state of decomposition, and fourthly, the use of an internal positive control effectively eliminated the chances of any false negative detection. Species specificity of all targets was confirmed by cross-checking all the primers and probes against 25 non-target species. The assay was sensitive enough to detect all the bovine, buffalo, and porcine targets in pure, admixed and processed frankfurter and meatball samples having as low as 0.1% adulteration.

Furthermore, the screening of the commercial food samples reflected that the assay could be applied for the analysis of real-world samples.

Thus, this novel assay demonstrated sufficient merits to be used by regulatory bodies for beef, buffalo, and pork authentication in any samples even under degraded conditions. In addition to multiplex, the assay could be used in simplex PCR system for identification of all targets individually as well as tetraplex PCR system for identification and differentiation of beef and buffalo under double targeted system based on the requirements and needs. A brief summary of the present research according to the objectives are given below:

1. Development of Biomarkers Targeting Double Genes Sites

The amplicon sizes of the designed three sets of primers for each of cow, buffalo and pig were between 73 and 146 bp (cytb and ND5 genes of cow (120 and 106 bp), buffalo (90 and138 bp), Pork (146 and 73 bp)). Biomarker targets within this range were suitable for efficient amplification and stability under extreme food processing conditions. The in-silico analysis revealed that the developed six primer sets matched 100% with the targets and had 3–18 nucleotides (12.5–78%) mismatching with non-target species, indicating that the primers would only bind with target DNA and avoid annealing with any potential non-targets. Moreover, measurement of pairwise distances and phylogenetic tree among the 24 animals and four plants species revealed sufficient genetic distances to rule out any chances of non-target amplification. Finally, the theoretical results were experimentally validated by an authentic PCR test against the target and 27 different non-target species using 20 ng of DNA extracted from all of the tested samples.

2. Development of multiplex conventional and real-time PCR systems

Mitochondrial cytb and ND5 genes were targeted and six different targets (length: 90-146 bp), two for each of cow (120 and 106bp), buffalo (90 and 138bp) and pig (73 and 146bp), were successfully amplified from raw, boiled, autoclaved and microwaved cooked meat under pure and mixed matrices. The detection limit was 0.01 ng DNA for tetraplex and 0.02 ng DNA for hexaplex under pure states and 0.1% target meat in mixed and commercial matrices. For authentication, the PCR products were digested by four restriction enzymes (RE), namely *Alu*I, *Eci*I, *Fat*I and *CviKI*-1 and clear restriction fingerprints were obtained. PCR products sequencing results confirmed the authenticity of RFLP findings. I also described here a quantitative multiplex real-time PCR assay with TaqMan Probes to detect and quantify contributions from bovine, buffalo and porcine materials simultaneously. Amplicon-sizes were very short (120, 90 and 146 bp for bovine, buffalo and porcine) because longer targets break down, bringing serious ambiguity in molecular diagnostics. False negative detection was eliminated through an endogenous control (141 bp site of eukaryotic 18S rRNA). Analysis of reference samples reflected good target recovery, PCR efficiency and detection limit under mixed matrices. The developed mqPCR assay successfully detected 0.003 ng DNA in a pure state and 0.1% target meat in mixed and commercial matrices.

3. Assay performance under processed meat products

The novel methods were used for the screening of bovine, buffalo and porcine materials in various commercial meat curries and processed foods, namely meatballs and frankfurters. Survey results revealed about 80% of beef meatballs were adulterated with buffalo and surprisingly about 20% the beef products were totally replaced with buffalo. Moreover, the analysis of 20 beef frankfurters revealed the presence of both beef and buffalo in all specimens. This demonstrated that all beef frankfurter products were

adulterated with buffalo in Malaysia. However, the analysis of 7 beef curries reflected only 2 them were beef and others 5 were buffalo. In contrast, porcine meatball and frankfurter were found 100% authentic and also no porcine was detected in halal branded beef curries, meatballs and frankfurters and also in chicken frankfurters. Furthermore, market survey under mqPCR assay revealed 71%, 100% and 85% of beef frankfurters, meatballs and 85% burgers contained buffalo adulteration but no porcine in Malaysian markets.

6.2 Recommendation for Future Work

The developed short amplicon length and double genes targeted multiplex food authentication schemes in the present study are highly promising technique as they can detect the targets in qualitatively and quantitatively in severely heat treated samples wherein most of DNA are degraded. Furthermore, the assay was highly secured as alternative targets could complement the detection of a missing target. In addition, quantification of the target DNA can be helpful to measure the limit of contamination.

Due to time limitation, all types of samples such as dairy and pharmaceutical products could not be analyzed. I believe this technique can be applied for the detection of species origin in pharmaceutical capsule shells, dairy and cosmetics samples as well. The sources of capsule shell gelatin are bovine, buffalo, porcine and fish. Among these bovine and porcine are most dominant sources but they have limited acceptability due to religious belief. However, the DNA extraction from capsule shells, dairy and cosmetics items is also difficult and has not been optimized yet; hence an appropriate protocol for the extraction of DNA should be developed. Furthermore, horse meat is another potential adulterant worldwide, since it has also limited acceptability because of potential zoonotic threats and religious restriction. If double genes targeted two horse biomarkers can be included to develop on octaplex system, it would be highly appreciated.

The current trend of the researchers to develop biosensor based techniques which offer fast, low cost, high sensitive that does not require expensive laboratory set up, costly instruments and skilled personnel and offer on-field delivery of results could be developed.

6.3 Limitation of this Study

It is difficult for another research group to replicate the results unless one has the capillary electrophoretic system like that of the automated Qiaxcel Advanced Capillary system. The nature of the study is the utilization of short length oligonucleotides which generate very small sized amplicons and differences between the amplicons were very short. Thus it requires special instrumentation for visualization. This incurs further costs. So, future study could explore whether such short-length variable amplicons could be separated by a different and more convenient approach.

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LIST OF PUBLICATIONS

1. **Hossain, M. M.**, Ali, M. E., Abd Hamid, S. B., Mustafa, S., Mohd Desa, M. N., & Zaidul, I. S. M. (2016). Double Gene Targeting Multiplex Polymerase Chain Reaction–Restriction Fragment Length Polymorphism Assay Discriminates Beef, Buffalo, and Pork Substitution in Frankfurter Products. *Journal of Agricultural and Food Chemistry*, *64*(32), 6343-6354.

2. **Hossain, M. M.**, Ali, M. E., Hamid, S. B. A., Mustafa, S., Desa, M. N. M., & Zaidul, I. S. M. (2017). Targeting double genes in multiplex PCR for discriminating bovine, buffalo and porcine materials in food chain. *Food Control*, *73*, 175-184.

3. **Hossain, M. M.**, Ali, M. E., Hamid, S. B. A., Hossain, S. A., Nizar, N. N. A., Uddin, M. N., ... & Akanda, M. J. H. (2017). Tetraplex PCR assay involving double gene-sites discriminates beef and buffalo in Malaysian meat curry and burger products. *Food Chemistry*, *224*, 97-104.
4. **Hossain, M. M.**, Ali, M. E., Sultana, S., Asing, Bonny, S. Q., Kader, M. A., & Rahman M. A. (2017). Quantitative Tetraplex Real-Time Polymerase Chain Reaction Assay With Taqman Probes Discriminates Cattle, Buffalo And Porcine Materials In Food Chain. *Journal Of Agricultural And Food Chemistry*, (*Just Accepted).*

CONFERENCE PROCEEDING

1. **Hossain, M. A. M.**, Ali M, E., Hamid, S. B. A. (2015). Design of Double Genes Targeted Multiplex PCR; The International Conference on Purity, Utility Reaction and Environmental Research – PURE2015 on the 9-11 November, 2015 in Kuala Lumpur, Malaysia.

2. **Hossain, M. A. M.**, Bhassu, S., Hamid, S. B. A., Ali M, E. (2015). Step by step development of double gene targeted multiplex PCR assay for the detection and differentiation of beef and buffalo in food chain; The 20th Biological Science Graduate Congress (20th BSGC) which will be held at Chulalongkorn University, Bangkok, Thailand.

PATENT

Dr. Md. Eaqub Ali, Professor Dr. Sharifah Bee Binti O.A Abd Hamid, **M. A. Motalib Hossain**. (2016). Double Gene Targetet Multiplex PCR Kit For The Detection And Differentiation Of Beef, Buffalo And Pork Derived Materials In Food Chain. Patent file no. PI 2016701716 and reference PIC/P/777/16/UM/Eaqub/1139-DG.

INTERNATIONAL SCIENCE NEWS

ACS News Service Weekly PressPac: Wed Aug 10, 2016: Frankfurter fraud: Finding out what's in your hot dog. https://www.acs.org/content/acs/en/pressroom/presspacs/2016/acs-presspac-august-10-2016/frankfurter-fraud-finding-out-whats-in-your-hot-dog.html